EXPLORING THE ROLE OF OESTROGEN IN CONTROLLING THE PATHOGENICITY OF *CANDIDA ALBICANS*

By

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Abstract

Candida albicans is an opportunistic human pathogen capable of causing mild to serious invasive infections in almost all human organs and tissue. Sex hormones are generally known to render the host susceptible to *Candida* infections due to their ability to regulate host immunity. For instance, oestrogen is a well-recognised risk factor for Vulvovaginal candidiasis (VVC). Uptake of oral contraceptives, pregnancy and hormone replacement therapy raises circulatory concentration of oestrogen thereby promoting VVC. Nevertheless, the mechanism behind the influence of oestrogen on VVC is unclear. The present study investigated how adaptation of *C. albicans* to oestrogen influences the fungal host-pathogen interaction. *C. albicans* adaptation to physiologically relevant concentrations of oestrogen led to reduced immune recognition, and enhanced virulence. This was facilitated via inhibition of opsonophagocytosis through increased recruitment of Factor H (FH) and complement components on fungal cell surface. Acquisition of FH and complement components occurred via Glycerol-3-Phosphate Dehydrogenase 2 (Gpd2), a *C. albicans* moonlighting protein. Oestrogen enhanced expression of *GPD2* through a non-canonical signal transduction pathway involving Bcr1. Thus, apart from rendering the host susceptible to VVC, oestrogen predisposes women to the infection by directly promoting fungal pathogenicity. Findings from this study may provide an opportunity to determine mechanisms behind gender-based predispositions to fungal infections and offer alternative strategies to improving health in women.

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ADCC	Antibody-dependent cellular cytotoxicity
Adh1	Alcohol dehydrogenase
AP-1	Activator protein 1
BSA	Bovine serum albumin
CLRs	C-type lectin receptors
CFW	Calcofluor White
C1-INH	C1 inhibitor
C4BP	C4 binding protein
CCP	Complement control proteins
DC	Dendritic cells
DMEM	Dulbecco's Modified Eagle Medium
DAF	Decay acceleration factor
DMSO	Dimethyl sulfoxide
E1	Estrone
E2	17β-estradiol
E3	Estriol
EE	17α-ethynylestradiol

EDTA	Ethylenediaminetetraacetic acid
EREs	oestrogen response elements
ERKs	Extracellular signal-regulated kinases
ECM	Extracellular matrix
EBP1	Oestrogen binding protein
Eno1	Enolase
ELISA	Enzyme-linked immunosorbent assay
Efb-C	Extracellular fibrinogen-binding protein
FBS	Foetal bovine serum
Fba1	Fructose-bisphosphate aldolase
FRT	Female reproductive tract
FACS	Fluorescence-activated Cell Sorting
FH	Factor H
FH-L	Factor H-Like
GUT	Gastrointestinally induced transition
Gpd2	Glycerol-3-phosphate dehydrogenase
Gpm1	Phosphoglycerate mutase 1
HS	Heparan sulphate

HRT	Hormone replacement therapy
Hsp90	heat shock protein 90
HMDM	Human monocyte-derived macrophages
KIRs	Killer-cell-Ig-like receptors
KOH	Potassium hydroxide
$KHCO_3$	Potassium bicarbonate
M-CSF	Macrophage Colony-stimulating Facto
MFI	Median fluorescence intensity
MCP-1	Monocyte chemoattractant protein-1
MPIα	Macrophage inflammatory protein
MCP	Membrane cofactor protein
MBL	Mannose binding lectin
MASPs	MBL-associated proteins
NETs	Neutrophil extracellular traps
NK	Natural killer cells
NCRs	Natural cytotoxicity receptors
NLRs	NOD-like receptors
NaCl	Sodium chloride

NH_4Cl	Ammonium chloride
NF-κB	Nuclear factor-kappa-B
PRRs	Pattern recognition receptors
PMNs	Polymorphonuclear neutrophils
PAMPs	Pathogen-associated molecular patterns
Pgk1	Phosphoglycerate kinase
PKC	Protein kinase C (PKC)
PBMCs	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PBS	Phosphate buffered saline
PTX	Pentraxins
Pdc11	Pyruvate decarboxylase
qPCR	Quantitative polymerase chain reaction
RLRs	RIG I-like receptors
RVVC	Recurrent vulvovaginal candidiasis
RAAS	Renin-angiotensin-aldosterone system
ROS	Reactive oxygen species

RPMI	Roswell Park Memorial Institute medium
SDA	Sabouraud's dextrose agar
SAPs	Secreted aspartic proteinases
Tal1	Transaldolase
TLRs	Toll-like receptors
VVC	Vulvovaginal candidiasis
WGA	Wheat germ agglutinin
YPD	Yeast peptone broth

1 Introduction

1.1 Medical importance of fungal pathogens

Though often neglected, fungal infections exert a significant burden on human health globally causing an estimated 1.5 million deaths each year (Almeida *et al.,* 2019). To date, approximately 2.2 to 3.8 million species of fungi have been described, but only 600 species are capable of causing human infections (Hawksworth *et al.,* 2017, Konopka *et al.,* 2019). Fungi that cause human infections predominantly belong to four groups namely the mucorales, ascomycetes, deuteromycetes and basiomycetes (Köhler *et al.,* 2017).

Fungal pathogens cause diseases in immunocompetent and immunocompromised individuals (Köhler *et al.,* 2015). The Entomophthoromycota, Basidiomycota and Ascomycota phyla comprise the majority of fungal pathogens that have an ability to infect healthy individuals (Köhler *et al.,* 2015). In the Basidiomycota, Ascomycota and Mucorales phyla are also some opportunistic fungal pathogens that target immunocompromised people (Köhler *et al.,* 2015).

Fungi cause a variety of human infections in different parts and organs of the body (Figure 1.1). Superficial skin infections are the most prevalent affecting nearly 1.7 billion people worldwide (Ameen 2010). Dermatophytes are the dominant cause of superficial infections causing common disease such as onychomycosis, athlete's foot and tinea capitis (Ameen 2010).

Oral and genital mucosa infections are also common, with more infections experienced in children and women respectively (Sobel 2007, Bongomin *et al.,* 2017). Generally, most superficial mycoses are treatable and not difficult to diagnose (Hay 2013).

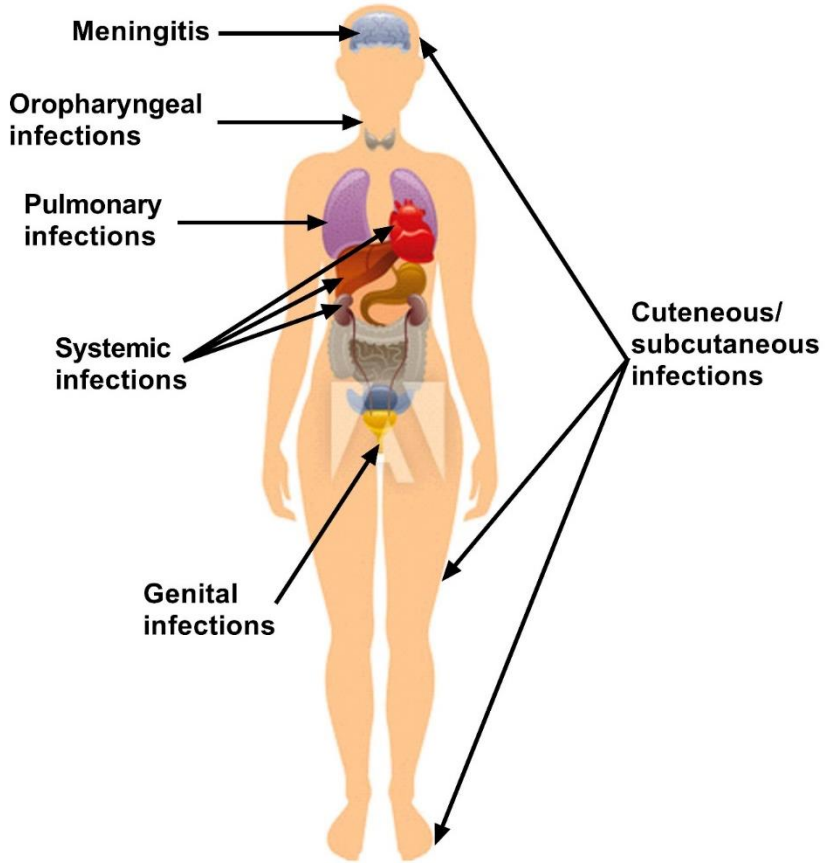

Figure 1.1. Anatomical sites and organs infected by fungal pathogens. Fungi cause infections on the skin, mucosal surfaces and in internal organs (Arenas et al., 2012).

Fungi also cause subcutaneous mycoses which frequently result from inoculation of the fungus into skin following trauma (Arenas *et al.,* 2012). These infections are common in tropical and subtropical regions of the world due to the hot and humid weather conditions (La Hoz *et al.,* 2012).

The most common subcutaneous infections are Chromoblastomycosis, Sporotrichosis and Eumycetomic (Arenas *et al.,* 2012). Subcutaneous infections may be difficult to treat but usually Sporotrichosis has a good prognosis (La Hoz *et al.,* 2012).

Systemic infections are either caused by opportunistic fungi or by invasive fungi also known as true pathogenic fungi due to their ability to invade and establish infection in tissues of healthy individuals (Arenas *et al.,* 2012, Rautemaa-Richardson *et al.,* 2017). The global incidence rates of systemic infections are much lower in comparison to other fungal infections, but they are associated with higher mortality rates. Systemic infections can develop from the lung following inhalation of the fungus or from endogenous microbiota resulting from damage or infection of the gastrointestinal tract and disseminate to other organs (Rautemaa-Richardson *et al.,* 2017).

Among opportunistic fungal diseases, candidiasis, aspergillosis, cryptococcosis, pneumocystosis and mucormycosis represent the most reported causes of invasive fungal diseases in the world (Bongomin *et al.,* 2017, Rautemaa-Richardson *et al.,* 2017). The most common invasive fungal diseases caused by true pathogenic fungi are blastomycosis, paracoccidioidomycosis, coccidioidomycosis, and histoplasmosis (Bongomin *et al.,* 2017, Rautemaa-Richardson *et al.,* 2017). *Candida* species remain common fungal pathogens causing a wide range of diseases.

In addition to systemic infections, *Candida* species commonly causes superficial infections. *Candida* species have virulence factors and attributes that enhance their ability to cause diseases and resist some antifungal treatment (Mayer *et al.,* 2013). Therefore, further research on *Candida* pathogenicity will help promote the development of new and improved diagnostic tools and treatment.

1.2 *Candida albicans*

1.2.1 Taxonomy

The fungal kingdom is comprised of eukaryotic organisms that exist in yeast and filamentous form. *C. albicans* belong to the genus *Candida* (Berman 2019) which contain about 200 species (Brandt *et al.,* 2012). The taxonomy of genus *Candida* has been changing over the years due to reclassification of some species as well as identification of new species (Daniel *et al.,* 2014).

1.2.2 Mating

C. albicans is predominantly a diploid fungus with a parasexual life cycle. The mating process in *C. albicans* has been previously described (Bennett *et al.,* 2005). First, *C. albicans* **a** and α white cells phenotypically switch to competent mating cells opaque **a** and α cells. Secretion of pheromones initiate the generation of mating projections which extend and allow the nuclei to migrate into the projections. The diploid **a** and α cells having opposite mating type fuse together to form a mating zygote.

The nuclei migrate towards each other and fuse. Daughter cell bud begins to form and then the nucleus divides across mother-daughter boundary. Septation develops to leave the daughter and mother cells with own nucleus. This is followed by formation of secondary buds.

1.2.3 Growth and morphology

Candida albicans has the capacity to grow in various morphological states such as yeasts, pseudohyphae, hyphae and chlamydospores (Whiteway *et al.,* 2007) (Figure 1.2). Yeasts are unicellular budding forms and hyphae or pseudohyphae are tubular structures. Hyphae cells are separated by septa and pseudohyphae lack separation (Whiteway *et al.,* 2007).

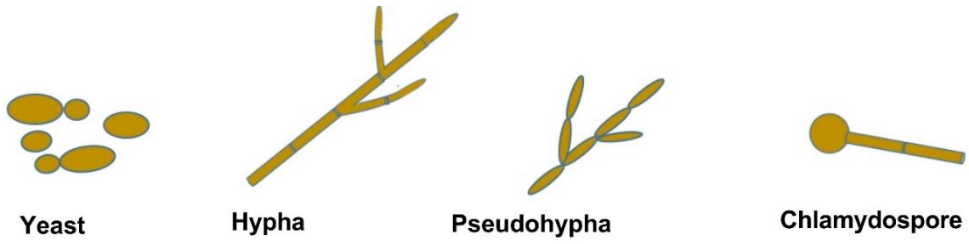

Figure 1.2. *C. albicans* morphologies. *C. albicans* can grow as budding yeast cells, pseudohyphae, hyphae and chlamydospores (Whiteway et al., 2007)

Chlamydospores are large spherical cells with a thick cell wall seen *in vitro* under particular adverse conditions including hypoxia and starvation (Staib *et al.,* 2007). In addition to the normal white and round-to-oval yeast cells, *C. albicans* can switch into other types of yeast cells. These include the opaque cells which are characteristically competent mating cells (Soll 2004), gastrointestinally induced transition (GUT) cells which are suitable for gut colonisation (Pande *et al.,* 2013) and grey cells which display an intermediate level of mating capability (Tao *et al.,* 2014).

Various host niches and cues such as low nitrogen, mannitol, 37˚C, CO_2, alkaline pH, amino acids, N-acetylglucosamine (GlcNAc), nutritional deprivation, hypoxia, peptidoglycan and hypercapnia are known to trigger morphological transition from yeast to hyphae (Noble *et al.,* 2017, Villa *et al.,* 2020). The Efg1-mediated cAMP pathway and the Cph1-mediated MAPK pathway are the common transduction pathways triggered by these signals to mediate transition to hyphal state (Noble *et al.,* 2017).

1.3 *Candida albicans* infections

There are nearly 200 species of *Candida* however only a few are of medical importance (Correia *et al.,* 2015). Among the few, *C. albicans*, a normal flora of the skin, mouth, oesophagus and vagina is the predominant cause of infections (Bongomin *et al.,* 2017). Predisposing factors to *C. albicans* infections include physical trauma, disturbance of normal flora due to overuse of broad spectrum antibiotics, and impaired immunity due to old age, HIV/AIDS, diabetes, leukaemia, chemotherapy, malnutrition and corticosteroids treatment (Singh *et al.,* 2014). *C. albicans* infections can be broadly categorised into superficial and systemic infections.

1.3.1 Superficial infections

Though quite common, superficial infections are mostly not fatal. However, they can cause discomfort and affect quality of life as well as act as a predisposition to invasive infections. Superficial candidiasis is mostly caused by *C. albicans*. Superficial infections include nail infections, cutaneous and mucosal infections.

Superficial infections can be diagnosed clinically and samples can be collected by swabbing or scrapping for laboratory confirmation of the fungus (Coronado-Castellote *et al.,* 2013).

Onychomycosis is a *C. albicans* nail infection characterised by proximal subungual infection, onycholysis and granuloma (Elewski 1998). In proximal subungual infection, the fungus infects the soft tissue around the nail and infiltrates the nail plate. The infected soft tissue looks red and swollen. The nail plate displays crosswise depressions called Beau's lines. Untreated nails lose shape and become rough and dystrophic. Onycholysis is characterised by distal subungual hyperkeratosis with an accumulation of a yellowish-grey substance that forces off the nail plate. *Candida* granuloma involves direct infection of the nail where its thickness is compromised. More severe cases where there are oedematous nail folds may lead to finger defect known as chicken drumstick (Elewski 1998).

Cutaneous candidiasis is a superficial infection of the skin. The common sites for cutaneous candidiasis are between the toes or fingers, below the gluteus, under the breasts, the groin area, and armpits. These sites are conducive for infection because they are warm and humid (López-Martínez 2010). Maceration, warm weather and obesity are other predisposing factors to cutaneous candidiasis (López-Martínez 2010). Patients with cutaneous candidiasis most commonly present with a rash, discharges, burning and rarely pain however they sometimes experience slight itching and develop whitish spots on infected areas (López-Martínez 2010).

C. albicans mucosal infections include oral candidiasis, oesophageal candidiasis, and vaginitis. Oral candidiasis patients present with white or erythematous lesions (Millsop *et al.,* 2016). While a patient with oesophageal candidiasis has yellow-white patches that can be distributed throughout the whole oesophagus or confined in the upper, middle, or distal part of the oesophagus (Klotz 2006). Symptoms of oesophageal candidiasis include pain behind the sternum, pain or difficulty in swallowing and rarely abdominal pain, weight loss, diarrhoea, nausea, vomiting, heartburn and melena (Rosołowski *et al.,* 2013, Alsomali *et al.,* 2017).

1.3.2 Systemic candidiasis

Systemic candidiasis can present as candidemia or deep-seated tissue infections. Most hospitals in developed countries report *C. albicans* as the most common cause of systemic candidiasis, however other parts of the world indicate that non-*albicans Candida* species could altogether contribute to over 50% of candidemia (Pappas *et al.,* 2003, Wisplinghoff *et al.,* 2004, McCarty *et al.,* 2016). Infections occur as a result of dissemination of *Candida* in the bloodstream to vital organs where they colonise and invade tissue or through direct inoculation of the fungus into the tissues following surgery or trauma (Lamoth *et al.,* 2018). Irrespective of antifungal treatment, the mortality rate of systemic infections is high reaching up to 40% (Wisplinghoff *et al.,* 2004, Arendrup 2013, Kullberg *et al.,* 2015). Systemic *Candida* infections can be diagnosed through blood culture, histology of infected tissue and PCR (Correia *et al.,* 2015) but their delay in diagnosis significantly increases mortality.

1.4 Immune responses against *C. albicans*

1.4.1 *C. albicans* Cell wall

The fungal cell wall acts as the first point of contact with immune defence as such it plays a key role in defining events associated with host-pathogen interaction. *C. albicans* cell wall is made up of two main layers. The outer layer is comprised of mannan, and the inner layer contains β-glucan and chitin (Figure 1.3). The two layers are covalently linked together by mannoproteins. The β-glucan-chitin matrix provides the shape and strength of the cell wall. β-glucan is the most abundant carbohydrate and cell wall component representing about 50-60% of the cell wall dry weight (Garcia-Rubio *et al.,* 2020) . β-glucan is comprised of glucose moieties that are connected through β-1,3 or β-1,6 linkages (Iorio *et al.,* 2008). β-1,6-glucan are in less amount than β-1,3-glucan.

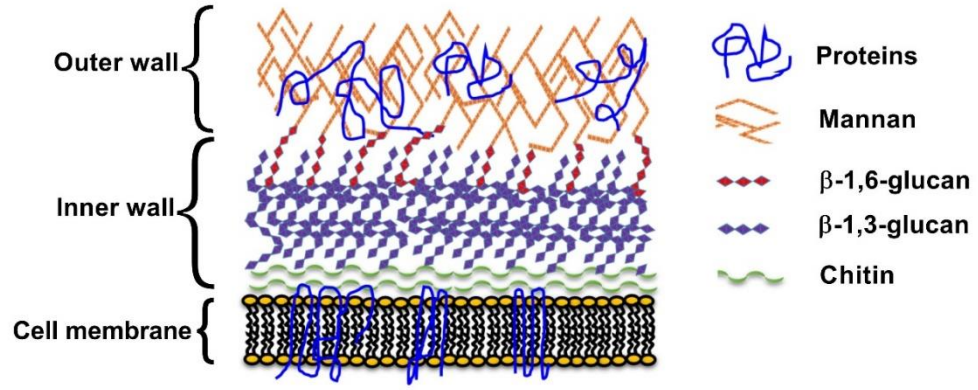

Figure 1.3. Structure, organisation, and composition of *C. albicans* cell wall. The outer layer of *C. albicans* cell wall contains highly mannosylated proteins that are cross-linked to β-(1,6) glucan which is attached to the inner layer composed of the β-(1,3) glucan-chitin core. The β-(1,3) glucan-chitin complex provides strength and shape of the cell wall whereas chitin present in the inner most part of the cell wall forms close-fitting antiparallel hydrogen-bonded structures linked with insolubility. Modified from Gow *et al*., (2017).

Together with chitin and mannoproteins, β-1,6-glucan covalently crosslink with the β-1,3-glucan skeleton in the inner layer of the cell wall thereby providing stability of the cell wall (Iorio *et al.,* 2008). The mannoproteins together with phospholipomannans make up 30-40% of the dry weight of *C. albicans* cell wall (Chaffin *et al.,* 1998). The mannoproteins in the outer surface of *C. albicans* cell wall are highly glycosylated with *O*-and *N*-linked oligosaccharides. Chitin is situated in the inner layer of the cell wall (Gow *et al.,* 2012) where it is are heavily interlinked and provide barrier to permeability to solutes (Chaffin 2008). Chitin constitutes only 1-2% of the dry weight of the cell wall but plays a significant contribution to the integrity of the cell wall (Garcia-Rubio *et al.,* 2020).

The *C. albicans* cell wall plays a critical role in host-pathogen interaction. The host is capable of recognising β-glucan, mannoproteins and chitin and trigger distinct immune responses against the fungus (Netea *et al.,* 2015). In addition, *C. albicans* cell wall is essential in promoting pathogenicity. The fungal cell wall promotes adhesion to host cells as well as invasion and physical damage of host tissue (da Silva Dantas *et al.,* 2016). Besides, *C. albicans* can remodel its cell wall in response to changes in the host microenvironment. Changes in host niches can trigger cell wall modulation in *C. albicans* and promote virulence of the pathogen (Hall 2015). Moreover, *C. albicans* adaptation to various host environments induce differential expression of cell wall proteins and subsequently influence host-pathogen interaction (Hall 2015). Nevertheless, little is known on how adaptation of *C. albicans* to oestrogen influence host-pathogen interaction.

1.4.2 Innate immunity

The innate immune system provides non-specific protection against infection. It includes physical and anatomical structures that inhibit entry of pathogens into the host as well as secreted molecules such as cytokines, antimicrobial peptides, complement proteins etc. There are several stages involved in launching a successful immune response, but the initial critical step performed by the innate immune system is the ability of the host to recognise the pathogen.

1.4.2.1 Innate immune cells

Innate immune cells involved in defence against *C. albicans* infections include, but are not limited to, epithelial cells, endothelial cells, dendritic cells (DCs), macrophages, monocytes, and natural killer cells. Epithelial cells act as the physical barrier and first line of defence against *C. albicans* infections. As discussed below, epithelial cells can detect the morphological changes of *C. albicans* and determine their commensal and pathogenic status as well as facilitate recruitment of immune cells to the site of infection. Whereas endothelial cells provide a secondary barrier to *C. albicans* infection and prevent entry of the pathogen into blood circulation. Moreover, endothelial cells express pattern recognition receptors (PRRs) (Zheng *et al.,* 2015) which detect *C. albicans* and trigger secretion of proinflammatory cytokines (Filler *et al.,* 1996, Orozco *et al.,* 2000, Müller *et al.,* 2007).

Dendritic cells (DCs) are innate immune cells generally known to function as the linkage between the innate and adaptive immune systems. DCs are less efficient at killing *C. albicans* (Netea *et al.,* 2004) however their main role is processing fungal antigens and presenting them to naïve T cells for their activation to fight infection (Ramirez-Ortiz *et al.,* 2012).

Tissue-resident macrophages are vital in defence against fungal infections. Macrophages detect *C. albicans* resulting in their activation and subsequent secretion of pro-inflammatory cytokines and chemokines that promote migration and stimulation of other immune effector cells to the site of infection (Goodridge *et al.,* 2007, Wells *et al.,* 2008, Krysan *et al.,* 2014). In addition, activated macrophages phagocytose and kill *C. albicans* through release of molecules such as ROS (reactive oxygen species) and RNS (reactive nitrogen species) (Qin *et al.,* 2016).

Monocytes are among the immune cells that are recruited to the site of infection where they differentiate into inflammatory macrophages. Monocytes have the ability to coordinate and mediate the innate and adaptive immune responses against fungi by regulating responses of NK cells, neutrophils, and T-cells (Hohl *et al.,* 2009, Domínguez-Andrés *et al.,* 2017). Tissue invasion and production of chemokines initiates recruitment of polymorphonuclear neutrophils (PMNs) to the site of *C. albicans* infection (Balish *et al.,* 1999, Huang *et al.,* 2004, Schaller *et al.,* 2004).

PMNs are critical in clearance of *C. albicans* infection and are considered the most potent killer of *C. albicans* having the ability to contain the fungus and prevent its development into hyphae (Brown 2011). PMNs can recognise *C. albicans*, phagocytose and kill it in a complex process involving the oxidative and non-oxidative strategies (Small *et al.,* 2018).

Natural killer (NK) cells recognise pathogens through natural cytotoxicity receptors (NCRs), killer-cell-Ig-like receptors (KIRs) and TLRs (Quintin *et al.,* 2013, Sivori *et al.,* 2014). The NKp30 receptor belonging to the NCR family recognises β1,3-glucan and triggers the PI3K and Erk1/2 signalling pathways. Subsequently, this leads to release of effector molecules including perforin, granulysin and granzymes (Orange 2008, Voigt *et al.,* 2014, Li *et al.,* 2018). Perforin perforates the cell membrane of the pathogen and induces lysis (Law *et al.,* 2010). Activated NK cells also produce cytokines and chemokines that consequently activate and enhance the antifungal activity of neutrophils (Bär *et al.,* 2014, Voigt *et al.,* 2014).

1.4.2.2 Innate immune receptors for sensing of *Candida albicans*

To recognise the pathogen, the innate immune system uses pattern recognition receptors (PRRs) to directly sense the preserved molecular components of the microbe known as pathogen associated molecular patterns (PAMPs). PAMPs exist in different forms such as carbohydrate, nucleic acid, protein, or lipid components. *C. albicans* is also indirectly detected by FcRγ and complement receptors once the fungus is opsonised with antibodies or complement, respectively. C-type lectin receptors (CLRs), RIG I-like receptors (RLRs), NOD-like receptors (NLRs) and Toll-like receptors (TLRs) make up the different families of PRRs with distinct roles in recognition of *C. albicans* PAMPs (Table 1.1).

1.4.2.2.1 C type lectin receptors

CLR family contain over 1000 receptors which are further classified into 17 subgroups based on their structural and domain characteristics (Zelensky *et al.,* 2005). CLRs can recognise the fungus directly or indirectly through association with the FcRγ receptor (Shiokawa *et al.,* 2017). This interaction results in stimulation of the Syk/PKCd/CARD9/Bcl-10/MALT1 signalling pathway and consequently the activation of relevant transcription factors to lead to the development of an immune response (Shiokawa *et al.,* 2017). However, other reports indicate that some CLRs operate through the RAF1 kinase pathway (Hardison *et al.,* 2012). Some of the notable CLRs that recognise *C. albicans* include Dectin-1, Mannose receptor, Dectin-2, Dectin-3, dendritic cell-specific ICAM3 grabbing non-integrin (DC-SIGN), Mincle, Galectin-3, collectins and Langerin (Stappers *et al.,* 2017).

Dectin-1 is a major CLR that senses fungal pathogens including *C. albicans* by recognising β(1,3)-glucan (Brown *et al.,* 2001). Dectin-1 facilitates phagocytosis of *C. albicans*, activation of reactive oxygen species (ROS), stimulation of pro-inflammatory cytokines and differentiation of Th17 cell (JA 2007, LeibundGut-Landmann *et al.,* 2007). Immune cells expressing Dectin-1 include neutrophils, macrophages and dendritic cells (Brown *et al.,* 2001). The Mannose receptor, Dectin-2, Dectin-3, dendritic cell-specific ICAM3 grabbing non-integrin (DC-SIGN), Mincle, Galectin-3, collectins and Langerin are examples of CLR that have been linked with recognition of *C. albicans* mannan and mannoproteins (Stappers *et al.,* 2017).

1.4.2.2.2 Toll-like receptors

There are 10 TLRs discovered in humans (Plato *et al.,* 2015). TLR1, 2, 4, 5, 6 and 10 are expressed on the cell surface whereas TLR3, 7, 8, and 9 are intracellular receptors (Kawai *et al.,* 2010, Celhar *et al.,* 2012). Cells known to express TLRs include monocytes, macrophages, dendritic cells, neutrophils, epithelial cells and T cells (Medzhitov 2007). TLR3 signals through TRIF-dependent pathway whereas all other TLRs signal via MyD88 (El-Zayat *et al.,* 2019).

TLR2, TLR3, TLR4, TLR7 and TLR9 are all important in recognising *C. albicans*. TLR2 can sense phospholipomannans in *C. albicans* and stimulate production of proinflammatory cytokines (Netea *et al.,* 2002, Jouault *et al.,* 2003, Gil *et al.,* 2006). Loss of function of TLR2 through polymorphism promotes recurrent vulvovaginal candidiasis (Rosentul *et al.,* 2014). Similarly, TLR4 detects *O*-linked mannan in *Candida* and activates secretion of proinflammatory cytokines (Netea *et al.,* 2006). Mice deficient in TLR4 are prone to systemic candidiasis (Netea *et al.,* 2002) and polymorphisms in TLR4 promote candidemia (Van der Graaf *et al.,* 2006). TLR3 is also important in detecting *C. albicans* infection as its genetic variation in monocytes causes reduction in production of IFNγ and increases susceptibility to cutaneous candidiasis (Nahum *et al.,* 2011, Nahum *et al.,* 2012). TLR7 senses *C. albicans* RNA to cause a reduction in production of IL-12 and lack of the receptor in mice leads to systemic infection (Biondo *et al.,* 2012).

Nevertheless, data on *C. albicans* TLR recognition needs to be examined with care as it is highly variable as shown by other reports which revealed that TLR2 TLR3, TLR4 and TLR9 are not essential in recognition of *C. albicans* (Netea *et al.,* 2004, Villamón *et al.,* 2004, Villamón *et al.,* 2004, Gil *et al.,* 2006, Sutmuller *et al.,* 2006, Plantinga *et al.,* 2012).

1.4.2.2.3 NOD-Like and Rig-I-like receptors

The nucleotide binding and oligomerization domain (NOD)-like receptors (NLRs) and the RIG-I (retinoid acid-inducible gene I)-like receptors (RLRs) are other families of intracellular innate immune receptors that detect *C. albicans.* Humans contain 22 NLRs classified into five subfamilies namely NLRA, NLRB, NLRC, NLRP, and NLRX (Ting *et al.,* 2008).

NLRs play a vital role in sensing various microbes, processing and presenting antigens to antigen presenting cells and also activating the inflammasome (Netea *et al.,* 2015). NOD2 is an NLR that belongs to the NLRC2 family and has been implicated in recognising *C. albicans* chitin and stimulate production of IL-10 (Wagener *et al.,* 2014). NOD2 senses *C. albicans* chitin in cooperation with mannose receptor and TLR9 to result into attenuation of inflammatory response (Wagener *et al.,* 2014).

RIG-I, laboratory of genetics and physiology 2 (LGP2) and melanoma differentiation factor 5 (MDA5) make up the RLR family (Patin *et al.,* 2019). MDA5 senses *C. albicans* hyphae as deletion of MDA5 in macrophages leads to reduced production of IFN-β (Jaeger *et al.,* 2015).

In addition, genetic variation in *MDA5* are linked to the increased susceptibility to systemic candidiasis (Jaeger *et al.,* 2015).

Table 1.1. A summary of some of the PRRs known to detect C. albicans PAMPs.

PRR	PRR Family	Location on cells	Target PAMP
Dectin-1	CLR	Surface	β-(1,3)-glucan
Dectin-2	CLR	Surface	High-mannose structures
Dectin-3	CLR	Surface	α-mannan
Mannose receptor	CLR	Surface, secreted	*N*-linked mannan
Mincle	CLR	Surface	α-mannan
DC-SIGN	CLR	Surface	N-linked mannan
Galectin-3	CLR	Surface, secreted	β-1,2-linked oligomannans
Langerin	CLR	Surface	β-glucan, mannan
SP-A	CLR	Secreted	Mannan
SP-D	CLR	Secreted	Mannan
MBL	CLR	Secreted	Mannan
TLR2	TLR	Surface	Phospholipomannan
TLR4	TLR	Surface	*O*-mannan
TLR7	TLR	Endosomal	Single-stranded RNA
TLR9	TLR	Endosomal	DNA
NOD2	NLR	Cytoplasmic	Chitin
NLRP3	NLR	Cytoplasmic	β-glucan and SAP2/6

1.4.2.3 The complement system

The complement system is an intricate innate immune system providing protection against pathogens including *C. albicans*. The complement system involves a series of inactive proteins present in blood circulation and tissue fluids. Most of the complement proteins are proteases which get activated after their proteolytic cleavage.

Pathogens activate the complement though three distinctive pathways namely the alternative, the lectin, and classical pathways (Figure 1.4) (Sarma *et al.,* 2011). *C. albicans* is capable of activating the complement through all the three pathways however the system is not able to form pores on the fungus due to its thick cell wall (Harpf *et al.,* 2020).

1.4.2.3.1 Alternative pathway

The alternative pathway is predominantly activated by spontaneous hydrolysis of C3 to generate C3b which binds to pathogens (Pangburn *et al.,* 1980, Tack *et al.,* 1980, Pangburn *et al.,* 1981, Bexborn *et al.,* 2008) or through interaction with properdin (Hourcade 2006, Kemper *et al.,* 2009) and microbial components such as carbohydrates (Winkelstein *et al.,* 1978), proteins (Selander *et al.,* 2006, Iwaki *et al.,* 2011) and lipids (Mold 1989). This triggers recruitment of Factor B which is cleaved by Factor D into Ba and Bb and participate in assembly of C3 convertase (C3bBb) (Volanakis *et al.,* 1996, Harboe *et al.,* 2008).

1.4.2.3.2 Lectin pathway

The lectin pathway is triggered by the binding of mannose binding lectin (MBL) or Ficolin to carbohydrate structures on surfaces of pathogens (Matsushita *et al.,* 2002). MBL and Ficolin are present as complexes with MBL-associated proteins (MASPs) in circulation (Endo *et al.,* 2015). Once bound to pathogens, MASPs linked with MBL or ficolins are conformationally transformed and activated to cleave C4 to C4a and C4b and C2 to C2a and C2b resulting into formation of the lectin pathway C3 convertase (C4bC2a) (Wallis 2007).

1.4.2.3.3 The classical pathway

The classical pathway is activated by deposition of immune complexes surface of microbes or non-self-antigens and foreign substances (Cooper 1985). The C1 multimeric complex made up of C1q, C1r and C1s binds to the Fc portion of IgM or IgG in the antigen-antibody complex via C1q leading to activation of C1r and C1s (Gaboriaud *et al.,* 2004, Diebolder *et al.,* 2014, Gaboriaud *et al.,* 2014). Subsequently, C1s degrades C4 and C2 to C4a, C4b and C2a, C2b respectively and initiate generation of the classical pathway C3 convertase (C4bC2a) (Gál *et al.,* 2002, Gregory *et al.,* 2003, Bally *et al.,* 2009, Brier *et al.,* 2010). Furthermore, pentraxins (PTX) have been shown to activate the classical pathway by binding to C1q and provide defence against pathogens (Meri *et al.,* 2019).

The three complement pathways converge with the generation of a C3 convertase which cleaves C3 to form C3b and C3a (Nesargikar *et al.,* 2012). C3b binds to the surface of the pathogen and functions as an opsonin to promote immune recognition and phagocytosis (Merle *et al.,* 2015). C3b also partake in formation of alternative pathway C3 convertase (C3bBb) which cleaves C3 and generates more C3b (Nesargikar *et al.,* 2012). C3b can be degraded into iC3b and C3d or binds to C3 convertase and generate C5 convertase which cleaves C5 to C5a and C5b (Sarma *et al.,* 2011). This leads to formation of the membrane attack complex where C6 and C7 bind to C5b followed by C8 and several molecules of C9 (Merle *et al.,* 2015). The membrane attack complex perforates cell membranes and creates pores that lead to lysis of target cells (Bubeck 2014).

C3a and C5a are anaphylatoxins that exhibit various inflammatory effector functions. They are important in activation of immune and non-immune cells expressing anaphylatoxins receptors C3aR and C5aR (Klos *et al.,* 2009, Klos *et al.,* 2013). They can facilitate recruitment of phagocytes to the site of inflammation (Sarma *et al.,* 2011). They initiate secretion of histamine from mast cells (El-Lati *et al.,* 1994) and basophils (Kretschmar *et al.,* 1993) thereby mediating vasodilation.

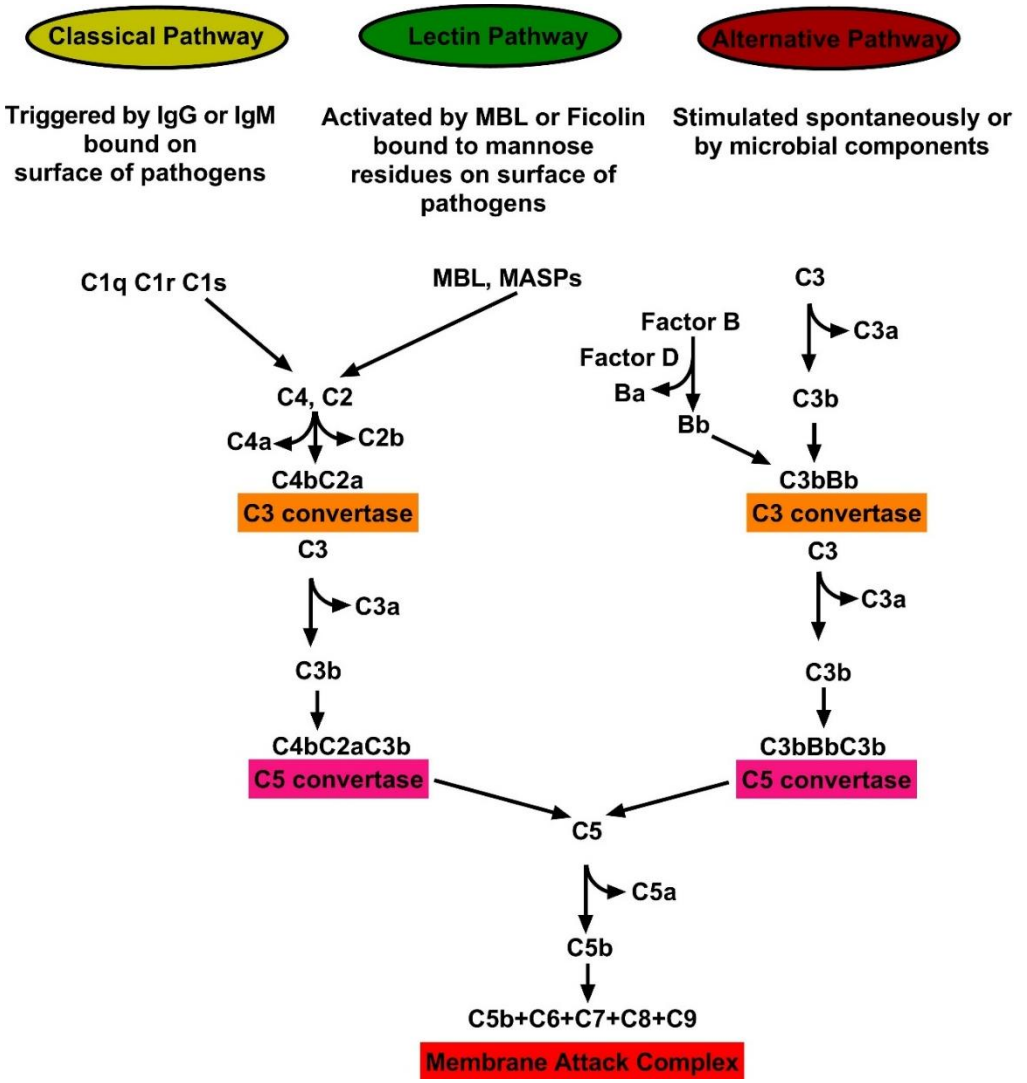

Figure 1.4. Activation of the complement system. The Complement system is made up of the classical, lectin and alternative activation pathways. Classical pathway is activated by deposition of immune complexes on surface of the pathogen resulting into association with the C1 complex. Lectin pathway is triggered by the binding of MBL or Ficolin to mannose residues on surface of pathogens resulting into activation of the MBLMASP complex. The classical and lectin pathways trigger formation of the classical C3 convertase C4b2a. Alternative pathway is stimulated by spontaneous hydrolysis of C3 into C3 (H2 O) leading to its cleavage to C3a and C3b which participates in formation of the alternative C3 convertase C3bBb. Complement activation leads to formation of the C5b-9 membrane attack complex and C3b deposition on pathogen surfaces thus promoting opsonisation and phagocytosis. It also induces inflammation by facilitating recruitment of immune cells and activation of endothelial cells, epithelial cells as well as platelets. Adapted from Sarma *et al*., (2011).

1.4.3 Regulation of complement activation

To avoid self-damage, the host cells produce various proteins and enzymes that regulate the effector functions of the complement system (Figure 1.5). The inflammatory effects of C3a and C5a are controlled through cleavage by plasma carboxypeptidases resulting in the reduction of their activity (Bokisch *et al.,* 1970). The activities of C3b and C4b are inhibited through proteolytic degradation by Factor I. In collaboration with Factor H, complement receptor 1 (CR1, CD35) and membrane cofactor protein (MCP), Factor I degrade C3b to iC3b (Davis *et al.,* 1984, Lambris *et al.,* 1996, Roversi *et al.,* 2011). CR1 further induces the cleavage of iC3b to C3c and C3dg (Lambris *et al.,* 1996). CR1 also binds to C3b in antigen-antibody complexes and mediate their phagocytosis otherwise existing immune complexes can be deposited on host tissue and activate the complement and cause tissue damage (Sarma *et al.,* 2011).

C1r, C1s and MASP2 are inhibited by C1-INH (Davis III *et al.,* 2008). The decay acceleration factor (DAF/CD55) hastens the decay of the classical and alternative C3 convertases (Lublin *et al.,* 1989, Seya *et al.,* 1989). In addition to preventing formation of the alternative pathway C3 and C5 convertase by competing with factor B for binding to C3b, Factor H also mediate the dissociation of convertases by dislodging bound factor Bb (Weiler *et al.,* 1976, Whaley *et al.,* 1976, Pangburn *et al.,* 1977).

Furthermore, S protein, CD59 and clusterin are known to interfere with the formation of the membrane attack complex and this in turn promote inhibition of the activity of the lytic complex (Podack *et al.,* 1984, Tschopp *et al.,* 1985, Farkas *et al.,* 2002). C4 binding protein (C4BP) inhibits formation of C3-convertase (C4bC2a) by acting as a Factor I cofactor in the degradation of C4b (Scharfstein *et al.,* 1978, Blom *et al.,* 1999). Along with inhibiting the generation of the classical C3-convertase by sequestering C4b, C4BP also accelerates the decay of C3 convertase (Gigli *et al.,* 1979).

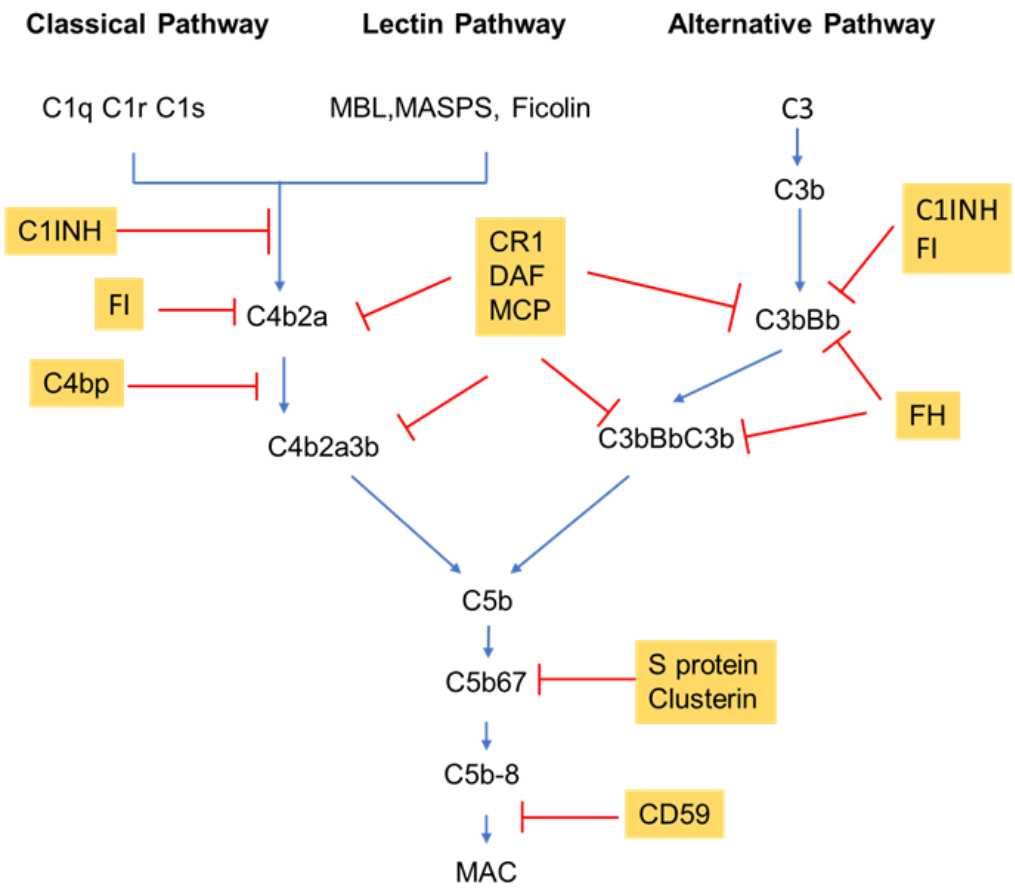

Figure 1.5 A schematic view of regulation of the complement system. Various complement regulators inhibit or control complement activation. C1 inhibitor (C1-INH) inhibits the activation of early pathway activation of all three pathways, whilst C4b-binding protein (C4BP) controls activation at the C4 level of the Classical and Lectin pathways. Factor I and factor H regulate the C3 and C5-convertases. Additionally, complement receptor 1 (CR1) and membrane cofactor protein (MCP) are the membrane-bound inhibitors that acts as co-factors for factor I and decay accelerating factor (DAF) which accelerates the decay of C3-convertases. The membrane-bound regulator Clusterin and CD59 prevents the generation of C5b-9, the membrane attack complex (MAC).

Taken together, the complement system recognises and provides immune defence against microbes through direct cell wall lysis, promotion of opsonophagocytosis, chemotaxis and activation of phagocytes (Merle *et al.,* 2015). Moreover, the complement system acts as a modulator of the adaptive immune system through augmentation of B cell responses and stimulation of T cell activation and migration (Morgan *et al.,* 2005). Nevertheless, *C. albicans* has developed various mechanisms to evade the complement system (Harpf *et al.,* 2020).

1.4.4 Adaptive immunity

1.4.4.1 T- Cell response

T-lymphocytes (T-cells) are vital cellular components proving protection against *C. albicans* infection. In response to *C. albicans* infection, Th1 secrete IFNγ that activate macrophages and enhance their ability to kill the fungus (Romani 1999). Lack of Th1 cells renders the host more prone to disseminated *Candida* infections since phagocytes cannot be properly activated to clear infection (Kullberg *et al.,* 1990, Netea *et al.,* 2003, Stuyt *et al.,* 2004). On the contrary, Th2 subsets are largely regarded as harmful since they seem to promote progression of fungal infections. For instance, secretion of IL-4 and IL-10 by Th2 prevents killing of *C. albicans* (Cenci *et al.,* 1993, Romani *et al.,* 1994). In addition, mice lacking IL-10 or injected with anti-IL-4R or IL-10R antibodies exhibited high resistance to invasive candidiasis (Haraguchi *et al.,* 2010).

Th17 cells secretes IL-17A, IL-17F and IL-22 which play a vital role in providing immunity against mucosal *C. albicans* infections. IL-17 mediates the recruitment and activation of neutrophils (Huang *et al.,* 2004) while IL-22 promotes strengthening the integrity of epithelial cells (De Luca *et al.,* 2010). Th17 cells confer immunity against oral mucosa and skin *Candida* infections (Kagami *et al.,* 2010, Hernández-Santos *et al.,* 2013). The role of IL-17 and IL-22 cytokines in protection against vaginal candidiasis is still unclear as there are conflicting results regarding its role (Yano *et al.,* 2010).

1.4.4.2 Humoral response

B-lymphocytes (B-cells) form an integral part of the humoral immune response by mediating the antibody-mediated immune responses. Though considered to play a minor role than cellular immune defence, antibody-mediated immune defence has proved to be important in providing protection against fungal pathogens. Antifungal humoral immune response activities that have been reported include, complement activation, opsonisation and antibody-dependent cellular cytotoxicity (ADCC) (Casadevall *et al.,* 2012, Casadevall *et al.,* 2012).

A few studies have demonstrated that antibodies can provide protection against *C. albicans* infection. For example, administration of monoclonal antibody (MAb) against mannan in mice provides protection from systemic candidiasis (Han *et al.,* 1995, Viudes *et al.,* 2004, Zhang *et al.,* 2006) and vaginal candidiasis (De Bernardis *et al.,* 1997, Han *et al.,* 1998).

Furthermore, growth and adhesion of *C. albicans* to human epithelial cells inhibits anti-β-glucan antibodies (Torosantucci *et al.,* 2005, Torosantucci *et al.,* 2009). Another investigation revealed that human antibodies against *C. albicans* heat shock protein 90 (Hsp90) confer protection in mice (Matthews *et al.,* 1991). Despite these advances, there is no vaccine available against *C. albicans* but a few glycoconjugates vaccines have been tested and they are in preclinical stage and yet to be verified for their safety and immunogenicity in humans (Costantino 2020).

Despite the robust immune defence mechanism in the host, *C. albicans* has developed immune evasion strategies which include morphogenesis, masking of PAMPs, complement inactivation, phenotype switching, inhibition of phagosome maturation, production of hydrolytic enzymes, manipulation of immune responses and inhibition of antimicrobial peptides activity (Stappers *et al.,* 2017) (Figure 1.6).

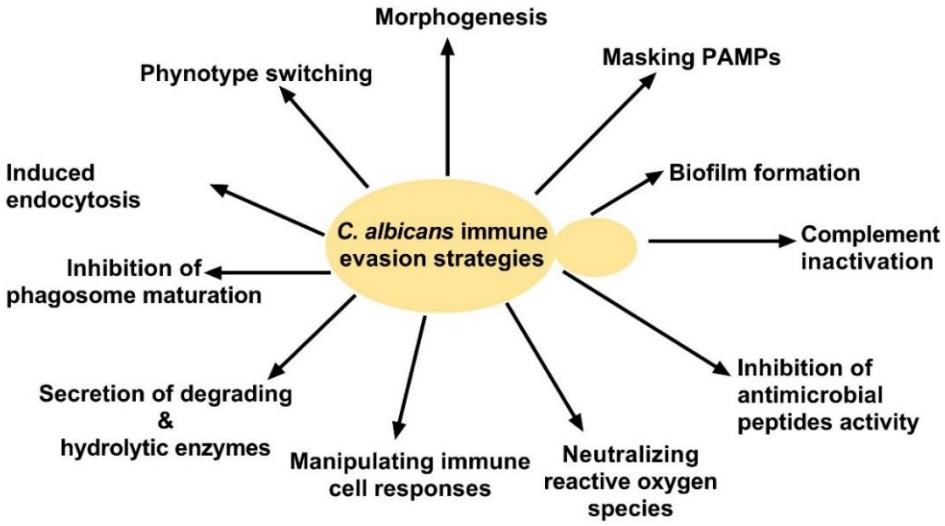

Figure 1.6 *C. albicans* immune escape mechanisms. The figure shows some of the mechanisms employed by *C. albicans* to evade immune effector functions (Stappers et al., 2017).

1.5 Moonlighting proteins

A moonlighting protein is a single polypeptide chain which normally has a conserved function, but it exhibits other biologically relevant biochemical or biophysical functions (Jeffery 1999). Interestingly, the multiple functions displayed by moonlighting proteins are normally not related (d Moore 2004). For instance, moonlighting proteins can have two distinct enzymatic functions, combine an enzymatic and non-enzymatic function, or have two non-enzymatic functions (d Moore 2004). Moonlighting proteins can execute various functions including as receptors, transcription factors, enzymes, adhesins etc (Jeffery 2014). *Candida species* express a few moonlighting proteins exhibiting various virulence attributes (Satala *et al.,* 2020).

In addition to their primary roles, most moonlighting proteins possess the capability to bind to host proteins and cells thereby enhancing adherence to host tissue. To mention a few, glyceraldehyde-3-phosphate dehydrogenase (Gpd2), alcohol dehydrogenase (Adh1), and phosphoglycerate mutase 1 (Gpm1) can bind to extracellular matrix (ECM) components and human plasminogen (HPG). ECM-binding and HPG-binding enhances pathogen attachment and tissue dissemination respectively thereby promoting fungal pathogenicity (Klotz *et al.,* 2001, Funk *et al.,* 2016). Enolase (Eno1), Gpm1, and Gpd2 are known to promote adhesion to endothelial cells (Luo *et al.,* 2013, Lopez *et al.,* 2014). Gpd2 and Gpm1 interact with components of the complement system and inhibit its activity (Poltermann *et al.,* 2007, Luo *et al.,* 2013).

Thus, investigating the role of moonlighting proteins on fungal virulence is important as it could help improve our understanding on the mechanisms behind pathogenesis of fungal diseases.

1.6 Vulvovaginal candidiasis

Vulvovaginal candidiasis (VVC) is a mucosal infection of the lower female reproductive tract (FRT). VVC is a common human *Candida* infection affecting nearly 75% of otherwise healthy women of reproductive age group (Sobel 2007). Nearly 8% of these women suffer at least three repeated infections of VVC in a year (Sobel 2007). VVC is mostly caused by *C. albicans*, but other *Candida* species such as *C. glabrata*, *C. tropicalis, C. parapsilosis* and *C. krusei* also cause the disease (Gonçalves *et al.,* 2016). Previous studies show that 1-10% of VVC infections are due to mixed infections most caused by *C. albicans* and *C. glabrata* (Richter *et al.,* 2005, Paulitsch *et al.,* 2006, Cetin *et al.,* 2007, Fan *et al.,* 2008, Amouri *et al.,* 2011, Rad *et al.,* 2011).

1.6.1 Signs and symptoms of VVC

C. albicans is one of the asymptomatic colonisers of the vaginal lumen (Achkar *et al.,* 2010). However, when the bacterial-fungal microbiota balance is physiologically or non-physiologically disturbed, *C. albicans* overgrows and triggers mucosal inflammation and invades the vaginal epithelium to cause symptomatic infection (Willems *et al.,* 2020). VVC is characterised by an influx of neutrophils in the vagina lumen, but with limited impact in clearance of the fungus (Fidel *et al*., 2004).

During infection, heparan sulfate in the vaginal lumen renders neutrophils ineffective as it competes with the phagocytes in interacting with *Candida* thus preventing killing of the fungus (Yano *et al.,* 2018). Clinical manifestations of VVC include pruritus, vaginal discharge, vaginal soreness, irritation, vulvar burning, dyspareunia, and external dysuria (Gonçalves *et al.,* 2016). VVC is not a life-threatening infection, however, it causes discomfort, pain, anxiety, psychological distress and impaired self-esteem and sexual relations (Sobel 2007).

1.6.2 Immune response

Like other body sites, the vagina environment has innate and adaptive immune strategies to combat infections. The vagina contains epithelial cells, dendritic cells (DCs), as well as T-helper cells, regulatory T cells, cytotoxic T cells, B-lymphocytes and natural killer cells which secrete chemokines and cytokines essential in recruiting extra immune factors from other sites (Piccinni *et al.,* 2002, Ghaleb *et al.,* 2003, Wira *et al.,* 2005, Wira *et al.,* 2005, Cole 2006, Fidel Jr 2007).

Filamentation and increased fungal burden are associated with symptomatic infection of the vagina. Thus, vaginal epithelial cells have developed a strategy to distinguish yeasts from hyphal cells via stimulation of distinct MAPK kinase pathways. The presence of low or tolerable numbers of *C. albicans* yeast cells stimulates a MAPK response characterised by activation of the c-Jun transcription factor (Moyes *et al*., 2011).

However, generation of hyphae and increased fungal burden triggers a MAPK response involving activation of the MKP1 and c-Fos transcription factor (Moyes *et al.,* 2011). Candidalysin, a hyphal related toxin is the major activation factor for the MAPK1 and c-FOS pathways (Moyes *et al.,* 2016). Once secreted, the protein is able to damage and stimulate epithelial cells (Moyes *et al.,* 2016).

Apart from providing a physical barrier to pathogens, epithelial cells have the ability to detect danger signals by pathogens and trigger a series of reactions which in turn stimulate the NLRP3 (NOD-, LRR- and pyrin domain-containing protein 3) inflammasome (Hise *et al.,* 2009, Bruno *et al.,* 2015, Roselletti *et al.,* 2017). Activation of the NLRP3 inflammasome leads to production of antimicrobial compounds, recruitment of neutrophils into the vagina lumen and secretion of IL-1β and IL-18 which are critical in activation of anti-*Candida* Th-cell subsets such as Th17 (Pietrella *et al.,* 2011, Roselletti *et al.,* 2017). Altogether, these defence strategies and subsequent immune effectors triggered, work to clear out *C. albicans* infection, however, if not well controlled, an exacerbated inflammatory response can occur and cause symptomatic infection called VVC (Willems *et al.,* 2020).

1.6.3 Diagnosis of VVC

Clinically it is challenging to diagnose VVC because most of the clinical symptoms are nonspecific since they are also related to other vaginal infections like trichomoniasis, bacterial vaginosis or gonorrhoea (Anderson *et al.,* 2004), as such it is important to confirm VVC with laboratory investigations. Vaginal swabs and particularly vaginal secretions are important samples for detecting of VVC infection. Samples are directly spread on a glass microscope slide and examined as a wet mount with 10% KOH or fixed dried, gram stained and examined using oil immersion objective (Cuenca-Estrella *et al.,* 2012).

Yeast cells, hyphae or pseudohyphae can be observed. Fungal selective agar media can be used to culture vaginal swabs or secretions to isolate *Candida* and confirm infection (Cuenca-Estrella *et al.,* 2012). Identification of species and performing susceptibility testing is vital in complicated cases, recurrent VVC or if the patient was previously treated with azole drugs. Nearly 20% of women have *Candida* species microbiota in the vagina (Bradford *et al.,* 2017) hence detecting *Candida* in culture in the absence of clinical manifestations of VVC is not an indication for antimycotic treatment (Workowski *et al.,* 2015). Other methods (e.g., PCR-based methods) for detection of VVC are available but most of the techniques are not validated.

1.6.4 Treatment of VVC

VVC is treated with oral therapy or topical agents. Uncomplicated cases are better treated with topical azoles than topical polyenes (i.e., nystatin) (Sobel *et al.,* 1995, Workowski *et al.,* 2015). Despite having potential adverse effects due to toxicity (Gonçalves *et al.,* 2016), oral azole drugs such as fluconazole and itraconazole have relatively higher cure rates than topical agents (Watson *et al.,* 2002). VVC infection with *C. glabrata* is generally difficult to treat with topical or oral azole agents as compared to VVC infection caused by *C. albicans* (Fidel *et al.,* 1999), due to the natural resistance of *C. glabrata* to azoles (Ferrari *et al.,* 2011). In addition, complicated infections including those caused by non-*albicans Candida* species are generally difficult to treat (Sobel *et al.,* 2001).

1.6.1 Predisposing factors to VVC

Host-associated risk factors that could render women prone to VCC include pregnancy, hormone replacement therapy, immunosuppression, uncontrolled or prolonged use of antibiotics and glucocorticoids, uncontrolled diabetes mellitus and genetic susceptibilities. In addition, some behavioural practices like using high oestrogen containing oral contraceptives, intrauterine device and condoms also increase the risk to developing VVC (Table 1.2). However, risk factors associated with recurrent vulvovaginal candidiasis (RVVC) are still unclear.

Table 1.2. Risk factors for VVC and their possible corresponding effects. Adapted from Gonçalves *et al*., (2016).

Host-related risk factors	Pregnancy Hormone replacement	Elevated levels of hormones promote *Candida* adhesion, development of hyphae and downregulation of vaginal immune responses
	Immunosuppression	Poor host immune response
	Antibiotics	Disturbance of the bacteria flora to *Candida* balance
	Uncontrolled diabetes Glucocorticoids	Elevated glucose levels weaken immune response and facilitate *Candida* adhesion to vaginal epithelial cells
	Genetic susceptibilities	Black women reported to be genetically susceptible
Behaviour-related risk factors	Oral contraceptives	Elevated levels of hormones promote *Candida* adhesion, development of hyphae and downregulation of vaginal immune responses
	Intrauterine device	Promotes *Candida* colonisation and biofilm formation which subsequently disseminate to cause infection
	Spermicides/condoms	Disturbance of the normal flora balance Metabolism of spermicides promotes *Candida* epithelial cell adhesion
	Poor personal hygiene Sexual habits	High burden of blastopore in the vagina area induces invasion of epithelial tissue by *Candida.*
	Clothing habits	Tight or non-cotton clothing increases levels of moisture and temperature in the perianal area hence promoting *Candida* growth.

1.7 Oestrogen in human development

The synthesis of sex steroid hormones involves hydrolysis of cholesterol esters followed by uptake of cholesterol by the mitochondria of specific tissue cells (Bremer *et al.,* 2014). Cholesterol is then converted to pregnenolone which is later metabolised to generate sex steroid hormones including oestrogen (Bremer *et al.,* 2014). In premenopausal women, oestrogen is predominantly produced by the ovaries, corpus luteum, and placenta while in men it is secreted by testes (Harvey *et al.,* 2014). The main physiological forms of oestrogen produced at different stages of steroid synthesis are estrone (E1), estradiol (E2, or 17β-estradiol), and estriol (E3) (Harvey *et al.,* 2014). 17β-estradiol is the principal product of oestrogen synthesis and it is the most potent oestrogen in premenopause whereas oestrone is vital in postmenopausal (Cui *et al.,* 2013). In addition, estriol is the least potent form of oestrogen largely useful during pregnancy (Cui *et al.,* 2013).

Oestrogen exerts a wide range of effects in mammals. Principally, oestrogen is essential in controlling and regulating reproduction, female development and sexual features (Reed *et al.,* 2015). In addition, oestrogen also regulates water and salt balance, enzyme production, skin and blood vessel elasticity, brain functions, blood fat levels and bone density and strength (Reed *et al.,* 2015). Oestrogen is critical in the development of the female reproductive tract organs such as the vagina, uterus and ovaries (Reed *et al.,* 2015).

During puberty, oestrogen facilitates the development of breast, growth of pubic hair, distribution of body fat, maturity of the mammary gland and growth of the nipples (Reed *et al.,* 2015). Furthermore, oestrogen has the ability to trigger as well as inhibit the adolescent bone-growth spurt (Cutler Jr 1997). The levels of oestrogen produced by the ovaries during the reproductive cycle vary at different times in response to environmental cues (such as concentration of other reproductive hormones) thereby inducing eggs to mature (Reed *et al.,* 2015). Oestrogen levels peak at ovulation stage following release of the egg by the ovary and thereafter the hormone levels drop to resume a new reproductive cycle (Figure 1.7) (Reed *et al.,* 2015). Nevertheless, once females reach the menopause stage, the concentration of oestrogen drops and the hormone no longer fluctuates episodically (Reed *et al.,* 2015). Consequently, the adrenal glands and other tissues responsible for converting androgens to oestrogen become the principal source of the hormone (Reed *et al.,* 2015).

Oestrogen is also essential in males as it regulates male fertility by influencing spermatogenesis, prostate development and function as well as modulating libido and erectile function (Schulster *et al.,* 2016, Hess *et al.,* 2018). Furthermore, oestrogen is known to control the development and maintenance of bones in young and adult males (Rochira *et al.,* 2015).

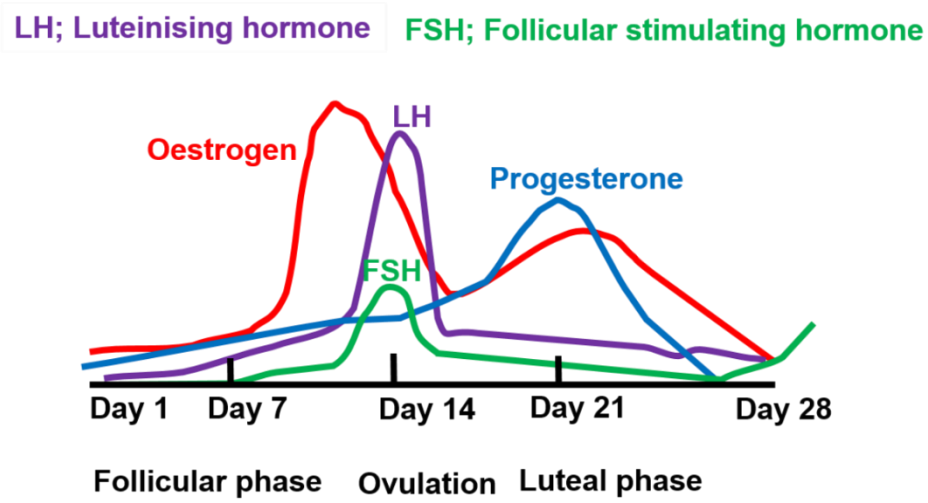

Figure 1.7. Fluctuating levels of oestrogen during the menstrual cycle. Oestrogen levels are secreted in large amounts during the follicular phase. The concentration of oestrogen drops during the ovulation phase, but the levels of luteinising hormone rise. The luteal phase is characterised by a surge in levels of progesterone and oestrogen. Adapted from Reed *et al*., (2015).

Oestrogen has also been reported to have harmful effects. Prolonged exposure to oestrogen promotes the risks of developing cancer of the breast, vagina and endometrium in women (Liang *et al.,* 2013). Excessive levels of oestrogen exacerbate endometriosis (Giudice 2010) whereas extremely low levels of oestrogen results into osteoporosis (Manolagas *et al.,* 2013). A poor balance in production of oestrogen in males can result into development of female characteristics and organs or diminished male features that could result into testicular cancer, poor sperm health and genital defects including hypospadias and cryptorchidism (Cooke *et al.,* 2017).

1.8 The role of oestrogen in pathogenesis of VVC

As reviewed by Gonçalves *et al.,* (2016), several studies have shown that oestrogen influences the progression of vaginal candidiasis (VVC). The mechanisms by which oestrogen influences VVC are still coming into view, as such there are some contradictory findings as regard to its role. Nevertheless, most studies agree that oestrogen is important in promoting pathogenesis of VVC. *In vivo* murine VVC infection models have shown that oestrogen is essential in establishment of infection as treatment of mice with oestrogen induces vagina infection and promotes persistence of infection (Fidel *et al.,* 2000, Hamad *et al.,* 2004). Furthermore, Fidel *et al*. (2000) revealed that oestrogen weakens the capacity of vaginal epithelial cells to control *C. albicans* growth. Similarly, it has been shown that higher levels of oestrogen in the vagina promote proliferation of *C. albicans* by providing more source of carbon through increased glycogen production (Dennerstein *et al.,* 2001).

Among other factors, pregnancy and use of hormone replacement therapy (HRT) and hormone-based contraceptives have been documented as predisposing factors of VVC (Sobel 2007). Increased incidences of VVC under these conditions is mostly linked to increased levels of sex hormones in circulation, including oestrogen. VVC infection rates are higher in the last trimester of pregnancy where the levels of oestrogen are elevated (Nelson *et al.,* 2013).

In non-pregnant women VVC is prevalent in the luteal phase of the menstrual cycle where levels of oestrogen are high (Kalo-Klein *et al.,* 1989, Eckert *et al.,* 1998). The importance of oestrogen in the pathogenicity of VVC is also demonstrated with the reduced incidences of the disease in pre-puberty and post-menopausal women, excluding those women on HRT (Bauters *et al.,* 2002, Tibaldi *et al.,* 2009).

Oestrogen regulates the function of immune cells, epithelial cells and fibroblasts in the female reproductive tract (FRT), however, its secretion also renders some parts of the FRT prone to infections (Wira *et al.,* 2015). Immune protection in the FRT is highly controlled such that immune responses differ depending on the stage of the menstrual cycle (Wira *et al.,* 2015). In order to optimise the environment for fertilization and pregnancy, the immune defence during the secretory phase of the menstrual cycle is diminished (Wira *et al.,* 2015). This generates an opportunity for possible pathogens such as *C. albicans* to cause infection in the FRT (Wira *et al.,* 2015).

Research indicates that progesterone stimulates the Cxcl1 gradient to promote neutrophil migration whereas estradiol interrupts the Cxcl1 gradient, hence promoting neutrophil arrest in the vaginal lining (Lasarte *et al.,* 2015). This might suggest that estradiol facilitates susceptibility to *C. albicans* infection in the vagina, while progesterone promotes protection because neutrophils are the main phagocytes responsible for clearance of *C. albicans*.

Recent work has also demonstrated that oestrogen inhibits neutrophils from killing *Candida* in the vagina by inducing production of heparan sulfate (HS) in the vagina (Yano *et al.,* 2018). Neutrophils utilises its receptor Mac-1 to bind to *Candida* and initiate the killing process (Yano *et al.,* 2018). Consequently, HS acts as a competitive ligand of *Candida* for Mac-1 on neutrophils leading to process known as neutrophil anergy (Yano *et al.,* 2018). Taken together, these studies highlight that oestrogen is vital in promoting development of VVC infection. However, more research is required to understand whether adaptation of *C. albicans* to the hormone can influence how it interacts with the host in the female reproductive tract.

1.9 Mechanism of oestrogen signalling

Oestrogen is the principal steroid hormone, which controls growth, differentiation, and function of several organs and tissues. Oestrogen initiates its activities by binding to oestrogen receptors (ERs) in the nucleus (Bjornstrom *et al.,* 2005). 17β-oestradiol (E2) is one of the most potent ligands of ERs. (ERs) are one of the nuclear transcription factors exhibiting ligan-dependent and ligand-independent transcriptional activity (Saha *et al.,* 2019). Once bound to oestrogen, ERs dimerise and bind to definite response elements known as Oestrogen Receptor Elements (EREs) (Nilsson *et al.,* 2001). EREs are expressed in the promoter regions of the target genes and among other things, binding with ERs triggers a conformational change within the binding region of the receptors (Nilsson *et al.,* 2001, Rosenfeld *et al.,* 2001).

The confirmational change triggers recruitment of activator proteins (Rosenfeld *et al.,* 2001). Using diverse strategies, ER regulates expression of several human genes. However, unlike EREs, ER does not contain sequences (O'Lone *et al.,* 2004). ERs facilitate gene expression without a need to bind to DNA directly. Receptors are linked by protein-protein interaction to a transcription factor complex to connect with DNA (Göttlicher *et al.,* 1998). ERE independent genomic activities are linked with interaction between ERs and activator protein-1 (AP-1) transcription factor complex. Unlike Erβ, ERα binds with SF-1 Receptor Elements (SFREs) (Vanacker *et al.,* 1999).

Interestingly, oestrogen displays little impact on gene expression, nonetheless their nongenomic activities are extremely fast and independent on activation of RNA and translation. These actions are facilitated via membrane linked ERs and by stimulating various protein kinase cascades (Lösel *et al.,* 2003). Nongenomic activities of oestrogen can indirectly stimulate gene expression by signal transduction activation pathways, that act on target transcription factors (Saha *et al.,* 2019). Protein kinase-mediated phosphorylation manages AP-1, and such transcription factors can be targets of oestrogen for nongenomic activities (Saha *et al.,* 2019). This is referred to as nongenomic to genomic signalling. Figure 1.8 provides a brief schematic overview of mechanism of oestrogen signalling.

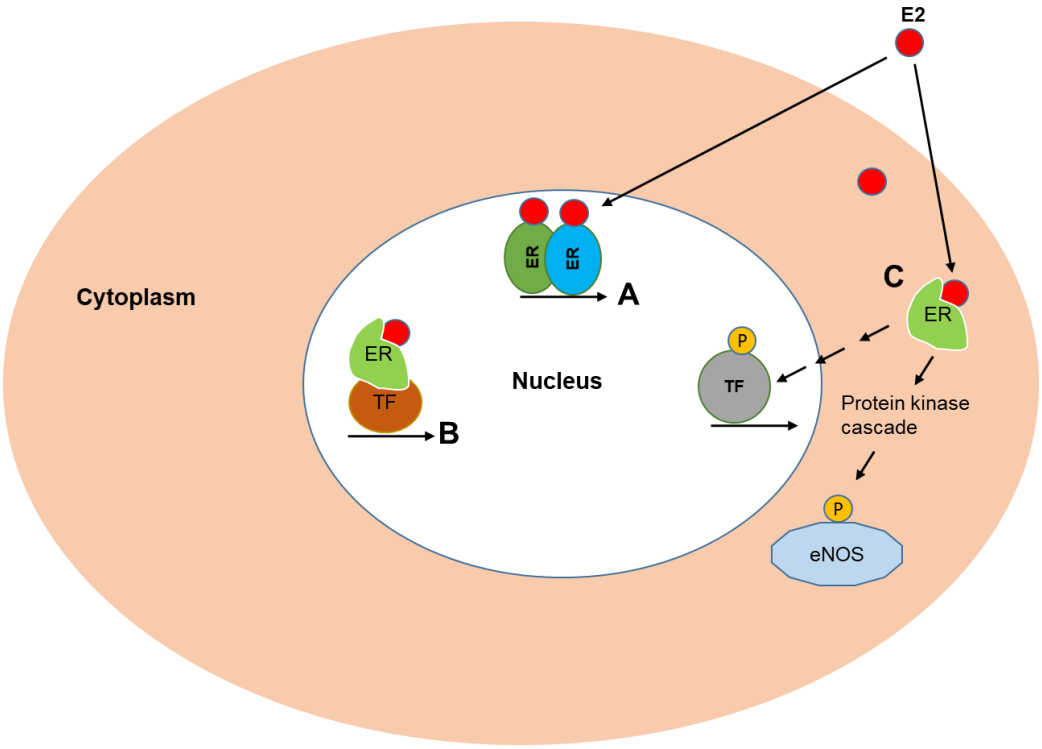

Figure 1.8. Oestrogen signalling. A. Classical mechanism: Nuclear E2-ERs directly bind to EREs in target gene promoters. **B. ERE independent genomic actions:** Nuclear E2-ERs complexes are secured by protein-protein interaction to a transcription factor (TF) complex that links with the target gene promoter. **C. Nongenomic actions:** Membrane E2-ER complexes stimulates protein kinase cascades leading to alteration of protein effector functions in the cytoplasm such as activation of endothelial nitric oxide synthase (eNOS), or to regulation of gene expression via phosphorylation (P) and activation of a TF. Adapted from Bjornstrom e*t al*., 2005.

1.10 Impact of oestrogen on *C. albicans*

It is generally accepted that oestrogen influence the pathogenesis of VVC and that sex steroids directly affect fungi in different ways. Previous reports have demonstrated the impact of oestrogen on *C. albicans* growth and morphology (Figure 1.9).

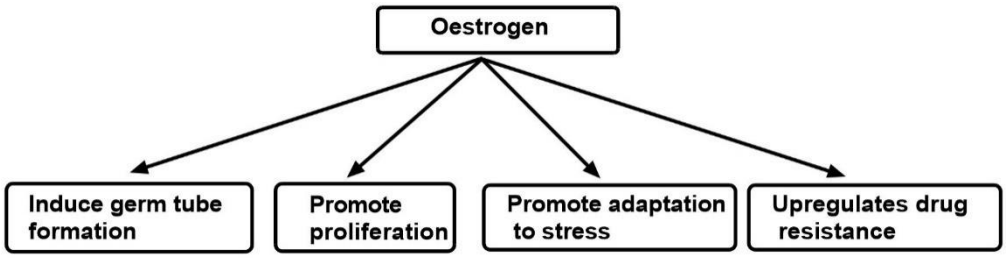

Figure 1.9. Effects of oestrogen on *C. albicans*. Figure shows different effects impacted by oestrogen on *C. albicans* (Gujjar *et al*., 1997, Zhang *et al*., 2000, Cheng *et al*., 2006, Kurakado *et al*., 2017).

The effects of oestrogen on *C. albicans* have been extensively studied due to the direct correlation of oestrogen levels and *C. albicans* vaginal colonization. Treatment of *C. albicans* with 17-β-estradiol induces germ tube formation in a dose- and strain-dependent manner (Cheng *et al.,* 2006). Induction of germination is due to increased expression of *PDR16* and *PDL1*, which promote morphogenesis. Filamentation is a critical virulent factor in *C. albicans* as it promotes colonization and invasion of the vaginal mucosa (Sobel *et al.,* 1984, Bernardis *et al.,* 1990). On the contrary, Kurakado *et al.,* (2017) revealed that 17β-estradiol inhibits formation of hyphae in *C. albicans*. Conflicting outcomes between Cheng *et al*. (2006) and Kurakado *et al*. (2017) regarding morphogenesis post oestrogen treatment could be attributed to the differences in their methodology. The two studies used different concentrations to evaluate filamentation. Varying concentrations of oestrogen exert different physiological effects. Unlike Cheng *et al.* (2006), Kurakado *et al.* (2017) used spider and *N*-acetylglucosamine media to induce hyphal formation and examine subsequent impact of oestrogen.

This could as well influence the outcome of the results since filamentation is already triggered by factor other than the hormone.

Zhang *et al.,* (2000) reported that oestrogen, promotes proliferation of *C. albicans*. This could be important in enhancing the rapid colonisation of *C. albicans* in the vagina. In addition, the study revealed that oestrogen enhances *C. albicans* adaptation to heat stress. Another study showed that supplementation of Sabouraud's dextrose agar (SDA) with 1 µM 17-β-estradiol promotes rapid growth of *C. albicans* (Gujjar *et al.,* 1997). Isolates cultured on SDA treated with 17-β-estradiol were larger than those growing on non-supplemented SDA media.

The varying reports on effects of oestrogen on *C. albicans* is proof that more research in this field is required, as this has the potential to improve our understanding on how *C. albicans* interact with the host. Besides, molecular evidence on how the fungi respond to human hormones in general is lacking and is an overlooked area of fungal biology.

1.11 Aim of the study.

1.11.1 Broad objective

1. To investigate whether elevated levels of oestrogen affect the interaction of *C. albicans* with innate immune cells.

1.11.2 Specific objectives

1. To determine whether oestrogen regulates the composition of *C. albicans* cell wall
2. To establish whether oestrogen regulate immune recognition of *C. albicans*
3. To ascertain how the adaptation of *C. albicans* to oestrogen is orchestrated.

2 Materials and Methods

2.1 Ethics

The ethical review board of the School of Biosciences at the University of Birmingham was responsible for approving all the protocols for human blood collection, isolation of peripheral blood mononuclear cells (PBMCs) and isolation of neutrophils (University of Birmingham ethics reference ERN_13-1487). Blood donors singed a written informed consent before donating blood. Collection of blood was done anonymously and on voluntary basis. Zebrafish care and experiment protocols were performed in accordance with the Home Office project license 40/3681 and personal license IE905E215 as per Animal Scientific Procedures Act 1986.

2.2 Strains and media

Unless specified otherwise, all media and chemicals were purchased from Sigma-Aldrich UK. All strains used in the study are listed in Table 2.1. *Candida* strains were routinely maintained on YPD agar (1% yeast extract, 1% peptone, 2% glucose and 2% agar). For broth cultures all strains were cultured in YPD (1% yeast extract, 1% bacto-peptone, 2% glucose). Oestrogen was diluted in 10% ethanol to a stock concentration of 1 mg/ml and diluted into YPD at the required concentrations, maintaining the final ethanol concentration at 0.3%.

Table 2.1. Candida strains used in this study

Strain	Genotype	Source/ reference
SC5314	Wild type	(Gillum *et al.,* 1984)
SVS006B	Vaginal clinical isolate	Prof Ramage, Glasgow University
SVS036A	Vaginal clinical isolate	Prof Ramage, Glasgow University
SN152	*arg4Δ/arg4Δ leu2Δ/leu2Δ his1Δ/his1Δ URA3/ura3Δ:: λimm434 IRO1/iro1Δ:: λimm434*	(Noble *et al.,* 2010)
SN250	*his1Δ/his1Δ, leu2Δ::C. dubliniensis HIS1 /leu2Δ::C. maltosa LEU2-arg4Δ/arg4Δ, URA3/ura3Δ::imm434-IRO1/iro1Δ::imm434*	(Noble *et al.,* 2010)
SN250-CIP30	As SN250 but *RPS1/rps1::*CIP30	Pizga Kumwenda/current study
*rob1*Δ	As SN152 but *rob1Δ::C. dubliniensisHIS1/rob1Δ::C. maltose LEU2*	(Noble *et al.,* 2010)
*bcr1*Δ	*arg4Δ/arg4Δ leu2Δ/leu2Δ his1Δ/his1Δ URA3/ura3Δ::λimm434 IRO1/iro1Δ:: λimm434 bcr1::LEU/bcr1::HIS1*	(Noble *et al.,* 2010)
*sfl1*Δ	As SN152 but *rob1Δ::C. dubliniensisHIS1/sfl1Δ::C. maltose LEU2*	(Noble *et al.,* 2010)
*rpn4*Δ	As SN152 but *rob1Δ::C. dubliniensisHIS1/rpn4Δ::C. maltose LEU2*	(Noble *et al.,* 2010)
*pho4*Δ	As SN152 but *rob1Δ::C. dubliniensisHIS1/pho4Δ::C. maltose LEU2*	(Noble *et al.,* 2010)

Strain	Genotype	Source/ reference
*tye7*Δ	As SN152 but *rob1Δ::C. dubliniensisHIS1/tye7Δ::C. maltose LEU2*	(Noble *et al.*, 2010)
*gal4*Δ	As SN152 but *rob1Δ::C. dubliniensisHIS1/gal4Δ::C. maltose LEU2*	(Noble *et al.*, 2010)
*ndt80*Δ	As SN152 but *rob1Δ::C. dubliniensisHIS1/ndt80Δ::C. maltose LEU2*	(Noble *et al.*, 2010)
*csr1*Δ	As SN152 but *rob1Δ::C. dubliniensisHIS1/csr1Δ::C. maltose LEU2*	(Noble *et al.*, 2010)
*ssn6*Δ	As SN152 but *rob1Δ::C. dubliniensisHIS1/ssn6Δ::C. maltose LEU2*	(Noble *et al.*, 2010)
*gpd2*Δ	As SN152 but *gpd2Δ::C. dubliniensisHIS1/gpd2Δ::C. maltose LEU2*	(Noble *et al.*, 2010)
*gpd2*Δ–CIP30	As SN152 but *gpd2Δ::C. dubliniensisHIS1/gpd2Δ::C. maltose LEU2, RPS1/rps1::CIP30*	Pizga Kumwenda/current study
*gpd2*Δ-CIP30-*GPD2*	As SN152 but *gpd2Δ::C. dubliniensisHIS1/gpd2Δ::C. maltose LEU2RPS1/rps1::CIP30-GPD2*	Pizga Kumwenda/current study
CAI4	*ura3::imm434/ura3::imm434 iro1/iro1::imm434*	(Fonzi *et al.,* 1993)
CAI4-pSM2	*ura3::imm434/ura3::imm434 iro1/iro1::imm434::*pSM2	(Hall *et al.,* 2010)
CAI4-pSM2-*GPD2*	*ura3::imm434/ura3::imm434 iro1/iro1::imm434::*pSM2-pTef2-*GPD2*	Rebecca Hall/current study
CAF2-1	*URA3/ura3:: λimm434*	(Sanglard *et al.,* 1997)
*cdr1*Δ	As CAF2-1, but *crd1::hisG/cdr1:hisG-URA3-HisG*	(Sanglard *et al.,* 1997)

Strain	Genotype	Source/ reference
cdr1Δ/cdr2Δ	As CAF2-1, but *crd1::hisg/cdr1:hisG, cdr2::hisG-URA3-hisG/cdr2::hisG*	(Sanglard *et al.,* 1997)

2.3 Tissue culture methods

2.3.1 Media and maintenance of cells

J774A.1 macrophages, NIH 3T3 fibroblasts, NIH 3T3-dectin-1 fibroblasts, A341 vaginal epithelial cells, human neutrophils, and human primary monocyte-derived macrophages (PBMCs) were used in the study.

The J774A.1 macrophage cell line was derived from a tumour in a female BALB/c/NIH mouse (Ralph *et al.,* 1975). It exhibits the adherence, cytological and phagocytic characteristics of macrophages (Ralph *et al.,* 1975). NIH 3T3 are adherent, fibroblastic and hypertriploid cells generated from NIH Swiss mouse embryo fibroblasts (Todaro *et al.,* 1963). NIH 3T3-dectin-1 fibroblasts, are NIH 3T3 cells expressing dectin-1, a specific receptor for β-glucans (Heinsbroek *et al.,* 2006). J774A.1 macrophages were maintained in complete DMEM media supplemented with 10% foetal bovine serum (FBS), 100 mM L-glutamine and 100 mM penicillin/streptomycin.

NIH 3T3 fibroblasts, NIH 3T3-dectin-1 fibroblasts and A341 vaginal epithelial cells were maintained in DMEM media supplemented with 10% heat inactivated FBS (56^{o}C for 30 min), 100 mM L-glutamine and 100 mM penicillin/streptomycin. PBMCs were cultured using adhesion media [500 mL RPMI 1640 with L-Glutamine (Gibco, 21875-091), 25 mL heat inactivated (56^{o}C for 30 min) pooled human AB serum (Sigma, Cat H4522-100ML), 100 mM penicillin/streptomycin (Sigma, P4333)]. Neutrophils were maintained in RPMI 1640 with L-Glutamine (Gibco, 21875-091), 100 mM penicillin/streptomycin (Sigma, P4333)].

2.3.2 Sub-culturing cells

To subculture J774A.1 macrophages, cells were passage into fresh media after reaching approximately 80 – 90% confluence. Procedurally, previous media was emptied and replenished with pre-warmed fresh complete DMEM media. Macrophages were mechanically scraped off the flask using cell scrappers. Cells were diluted into fresh media in tissue culture flasks. Cells were incubated at 37^{o}C, 5% CO_2.

To subculture NIH 3T3, NIH 3T3-dectin-1 and A341 cells, media was discarded, and cells were rinsed with PBS. Next, 1 mL of Trypsin-EDTA (0.25% Trypsin, and 0.53 mM EDTA) was added to the flask and incubated at 37^{o}C, 5% CO_2 for less than 10 min until cells are dislodged from surface of the flask. The flask is gently agitated, and 8 mL of media is added to neutralise the trypsin.

Cells are transferred into a 15 mL falcon tube and centrifuged at 2000 rpm, 20^{o}C for 5 min. Supernatant was discarded and cells were suspended in fresh media. Cells were sub-cultured into new flasks at a ratio of 1:10 or 1:20.

2.3.3 Freezing down cells for storage

Cells were grown in T75 tissue flasks until reaching approximately 80-90 % confluence. Cells were microscopically analysed for contamination by bacteria and fungi. Old media was removed and replaced with 10 mL of fresh complete DMEM media. Cells from 10 flasks were detached with cell scrappers and pooled into a 50 mL falcon tube. Cells were spun at 2000 rpm, 20^{o}C for 5 min. The supernatant was removed, and cells were suspended in 10 mL of freezing media made up of 50% foetal bovine serum (FBS), 40% complete DMEM and 10% Dimethyl sulfoxide (DMSO). The cell suspension was mixed, and 1 mL aliquots were added into 2 mL cryovials. Cryovials were quickly put into a Mr. Frosty™ Freezing Container and frozen overnight at -80 ^{o}C. Thereafter, cryovials were transferred into liquid nitrogen container for indefinite storage.

2.3.4 Recovery of cells from a liquid nitrogen storage container

The stored cryovials were carefully removed and briefly thawed in a water bath at 37^{o}C. Cells were quickly pipetted into a sterile T75 flasks containing 14 mL of complete DMEM media and incubated at 37^{o}C and 5% CO_2.

2.4 Isolation of monocytes and neutrophils from human whole blood

Isolation of monocytes and neutrophils was performed as previously described (Sherrington *et al.,* 2017). Using a serological pipette, 6 mL of percoll (Sigma Aldrich, GE17-0891-01) with density 1.079 (19.708 mL percoll, 11.792 mL water, 3.5 mL 1.5 M NaCl, filter sterilize) was added to 50 mL falcon tubes. 6 mL of percoll with density 1.098 was layered under percoll with density of 1.079 (24.823 mL percoll, 6.677 mL water, 3.5 mL 1.5 M NaCl, filter sterilize). Dual gradient was confirmed by eye. Gently, 6 mL of undiluted whole blood was layered over the dual gradient. The preparation was centrifuged for 8 min at 150 g, followed by 10 min at 1200 g with acceleration and break set to zero. White cells were separated into two layers; the top band were the monocytes/lymphocytes and the second band contained neutrophils. With care to avoid disrupting the rest of the gradient, monocytes and neutrophils were gently collected into respective 50 mL falcon tubes using a plastic pasture pipette. RBC lysis buffer (8.3g NH_4Cl, 1g $KHCO_3$, 0.04g Na_2 EDTA $2H_2O$, 2.5g BSA, per litre, filter sterilized) was added to the tubes containing neutrophil and monocytes at a ratio of 1:3. Tubes were gently inverted for 3 min and later centrifuged at 400 g for 6 min. Buffer was discarded and cells were washed twice in 40 mL PBS (ice cold PBS for monocytes and room temperature PBS for neutrophils) by centrifugation at 400 g for 6 min. Neutrophils were suspended in 1 mL RPMI media by gentle pipetting.

Cells were counted and re-suspended to 1 x 10^5 cells/mL and immediately used for phagocytosis experiments. Monocytes to be used for stimulation with *Candida* cells for cytokine production were re-suspended in RPMI at 2.5 x 10^6 /mL and 100 µL was added to 96 well plate. PFA fixed *Candida* cells were re-suspended to 0.5 x 10^6 preparation cells/mL in RPMI and 100 µL was added to the monocyte suspension in 96 well plate. The plate was incubated at 37^{o}C, 5% CO_2 for 24 h followed by spinning at 1200 rpm for 5 min and supernatant pipetted into a fresh 96 well plate. The supernatant was kept at -20 ^{o}C until ready to perform ELISA.

2.4.1 Differentiation of human peripheral blood mononuclear cells to primary macrophages

Primary macrophages were differentiated as previously described (Sherrington *et al.,* 2017). Monocytes were isolated as described above. Monocytes were re-suspended in 1 mL adhesion media [500 mL RPMI 1640 with L-Glutamine (Gibco 21875-091), 5% heat inactivated (56ºC for 30 min) pooled human AB serum (Sigma, Cat H4522-100ML), 5 mL Pen/strep (Sigma P4333)]. Monocytes were counted using haemocytometer and trypan blue to determine viability. Cells were diluted to 2.5 x 10^6 /mL in RPMI and 400 µL cells (0.5 x 10^6 /mL) were seeded in 24-well plates. Cells were incubated at 37^{o}C, 5% CO_2 for 1h to allow adherence to the wells. Monocytes were washed three times with cold PBS to remove non-adhered cells.

Next, 1 mL differentiation media [500 mL RPMI 1640 with L-Glutamine (Gibco 21875-091), 10% heat inactivated (56 ºC for 30 min) pooled human AB serum (Sigma, Cat H4522-100ML), 5 mL Pen/strep (Sigma P4333, M-CSF 20 ng/mL (Sigma SRP3110)] was added to the wells. Cells were incubated at 37 ºC, 5 % CO_2 for three days. Media was removed and replaced with fresh differentiation media and incubated for another three days. On day seven, macrophages were ready for phagocytosis experiments.

2.4.2 Phagocytosis experiments

2.4.2.1 J774A.1 macrophage phagocytosis

Phagocytosis experiments were performed as previously descried (Sherrington *et al.,* 2017) but with a few changes. Macrophages were seeded in 24-well plates at 1 x 10^5 cells /mL in DMEM media (500 mL DMEM, 50 mL FBS, 100 mM L-glutamine and 100 mM penicillin/streptomycin) with coverslips placed at the bottom. Cells were incubated overnight at 37 °C, 5 % CO_2. A 10 mL overnight *C. albicans* culture was also set on this day. Next day, *Candida* cells were harvested, washed, and suspended in PBS at 10 x 10^6/mL. J774A.1 macrophages were activated for 1 hr by replacing media with 1 mL serum free DMEM containing 1.5 µg/mL phorbol 12-myristate 13-acetate (PMA) (Sherrington *et al.,* 2017). Media on macrophages was replaced with 1 mL of fresh serum free DMEM and 50 µL of 10 x 10^6/mL yeast cells were added [Multiplicity of infection (MOI) = 5] and incubated for 45 min at 37 °C, 5 % CO_2.

Media was removed, cells washed twice with 1 mL PBS and fixed with PFA at room temperature for 20 min. PFA was removed and cells washed twice with 1 mL PBS. To distinguish between attached and internalised *Candida* cells, slides were stained in the dark with 200 µL ConA-TRITC 50 µg/mL for 30 min at room temperature followed by washing three times with 1 mL PBS. Coverslips were rinsed in distilled water and put on glass slide for imaging on Nikon Eclipse Ti microscope using 20x objective with 0.7x de-magnifier. Images were analysed using ImageJ software to score number of phagocytosed *C. albicans* cells. Non-stained *Candida* cells were scored as phagocytosed while stained yeast cells were considered as not phagocytosed. For each biological repeat and experimental condition, at least three images were scored and at least 200 macrophages were analysed. After scoring phagocytosed *Candida*, phagocytic index (PI) was calculated as follows:

$$\text{PI} = \frac{\text{Number of Phagocytosed } Candida}{\text{Number of analysed macrophages}} \text{ x } 100.$$

Where necessary, J774A.1 cells were maintained in DMEM supplemented with heat inactivated serum and the assay was complemented with either 2.5 µg/mL C3 (Sigma, C2910) or 1 µg/mL C3b (VWR International, 204860) and 1 µg/mL plasminogen (Sigma, 10874477001).

2.4.2.2 Human primary macrophage phagocytosis

Phagocytosis experiments were performed as described above except that following differentiation of PBMCs to primary macrophages, cells were not activated prior to phagocytosis.

2.4.2.3 Human neutrophil phagocytosis

Phagocytosis assays were performed as described in Section 2.4.4.1 above but with a few modifications. Following isolation, neutrophils were seeded in 24-well plates at 2 x 10^5 cells/mL and incubated for 1 h at 37°C, 5% CO_2 to allow cells to settle down. Without replacing media, 1 x 10^6/mL yeast cells were added and incubated for 45 min at 37°C, 5% CO_2. Thereafter, media was gently pipetted along the side of the wells and cells immediately fixed with PFA without washing. Washing cells would clear out most neutrophils as they do not adhere to the plates. Cells were imaged and analysed as described above.

2.4.3 Association of oestrogen-adapted *C. albicans* cells to fibroblasts

NIH 3T3 and NIH 3T3 Dectin-1 fibroblast cells were separately seeded in 24-well plates at 1 x 10^5 cells /mL in DMEM media with coverslips placed at the bottom and incubated overnight at 37 °C, 5 % CO_2. *Candida* cells were harvested, washed, and suspended in PBS at 10 x 10^6/mL. Media on fibroblasts was replaced with 1 mL of fresh serum free DMEM and 50 µL of 10 x 10^6/mL yeast cells were added (MOI = 5) and incubated for 45 min at 37 °C, 5 % CO_2. Media was removed, cells washed twice with 1 mL PBS and fixed with 4% PFA at room temperature for 20 min. PFA was removed and cells washed twice with 1 mL PBS. Coverslips were put on glass slide for imaging on Nikon Eclipse Ti microscope using 20x objective with 0.7x de-magnifier.

Images were analysed using ImageJ software to score number of *C. albicans* cells attaching to/associated with the fibroblasts. The average number of associated *Candida* cells per 200 fibroblast cells was determined.

2.4.4 Quantitative analysis of *C. albicans* adhesion to vaginal epithelial cells

Using a 24-well plate, 1 mL of 2 $x10^5$ A-431 cells in DMEM media were added to each well and cultivated for 2 to 3 days at 37°C, 5 % CO_2 until they reached 100 % confluency. *C. albicans* cells from overnight culture was diluted 1:100 in 10 mL of YPD and incubated for 4 h at 37 °C, 5% CO_2. *Candida* cells were harvested, washed three times with PBS and diluted to 6 x 10^3/mL. DMEM was removed from 24-well plates with A-431 monolayer cells and replaced with 1 mL of serum free DMEM for 2 h incubation at 37 °C, 5 % CO_2. Serum free DMEM added to test wells was supplemented with 10 µM and 0.001 µM 17 β-estradiol, respectively. After 2 h incubation, media was replaced with 250 µL fresh serum free DMEM (17 β-estradiol supplementation was maintained in relevant wells). Thereafter, 50 µL of 6 x 10^3/mL *Candida* cell suspension was applied to each well (i.e., 300 *Candida* cells per well). *C. albicans* cells added to A-341 cells include YPD control cells and oestrogen adapted cells treated with 10 µM and cells treated with 0.001 µM 17β-estradiol, respectively. YPD control cells were also added to wells containing media supplemented with E2. For each condition, *Candida* adhesion assay was performed three times in duplicates at three time points (0 min, 60 min and 120 min).

At time point 0 min, media was immediately removed soon after addition of *Candida* cells to the well. The removed supernatant was plated on YPD agar and incubated overnight at 30 °C. Colony forming units (CFUs) determined represented non-adherent cells at 0 min. Thereafter, 300 µL PBS was added to the well and adherent cells were scratched off using a 1-mL pipette. Next, 50 µL of the scratched-off cells were plated on YPD agar plates and incubated at 30 °C overnight. CFUs determined represented adherent cells at 0 min. Similar analysis was performed for time points at 60 min and 120 min. CFUs for all conditions were determined as such and percent adherence of *C. albicans* to A-341 cells was determined as follows:

$$\frac{\text{number of adherent cells}}{\text{number of adherent cells} + \text{number of nonadherent cells}} \times 100.$$

2.5 Enzyme-linked Immunosorbent Assay (ELISA)

2.5.1 Quantification of human cytokines

R&D Systems Human IL-6 DuoSet ELISA (DY206-05), Human IL-1 DuoSet ELISA (DY201-05), Human IFN-gamma DuoSet ELISA (DY285B-05) and Human TNF-alpha DuoSet ELISA (DY210-05) kits were used to quantify IL-6, IL-1β and TNF-α secreted from PBMCs co-incubated with *C. albicans* cells adapted to 17 β-oestradiol.

Locally prepared solutions used for the assays included PBS [137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.2-7.4, 0.2 µm filtered], TBS [20 mM Tris base, 150 mM NaCl, pH 7.2-7.4, 0.2 µm filtered], Wash Buffer [0.05 % Tween 20 in PBS, pH 7.2-7.4], Block buffer/reagent diluent [1 % BSA in PBS pH 7.2-7.4, 0.2 µm filtered], Substrate Solution [10 ml substrate buffer, 1 TMB tablet, 2.5 µL 30 % H_2O_2], Substrate buffer [3.55g Na_2HPO_4, 2.4g Citric acid, 500 mL H_2O, pH5, 0.2 µm filtered], Stop Solution [2N H_2SO_4]. All tests were performed as per manufacturer's instructions and measurements were done using FLUOstar Omega plate reader.

2.6 Cell wall immunofluorescent staining

C. albicans cells were stained as previously described (Sherrington *et al.,* 2017). Briefly, *C. albicans* cells from an overnight culture were grown at 37°C, 200 rpm for 4 h in YPD broth with or without oestrogen supplementation. Cells were harvested by centrifugation, washed in PBS three times, and fixed on ice with 4% paraformaldehyde (PFA) in PBS for 30 min.

To stain for total mannan, glucan and chitin levels in the cell wall, 2.5 x 10^6 *Candida* cells were separately stained on ice with 50 µg/ml tetramethyl rhodamine isocyanate (TRITC)-conjugated concanavalin A (Molecular Probes, Life Technologies) for 30 min, 33.3 µg/ml Aniline Blue fluorochrome (Bioscience supplies) and 3 µg/ml Calcofluor White (CFW) for 10 min.

To stain for exposed β1,3-glucan, PFA fixed 2.5 x 10^6 *Candida* cells were blocked on ice in 2% BSA in PBS for 30 min. Cells were spun down, supernatant discarded and re-suspended in 3μg/mL FC-dectin1 (a gift from G. Brown, University of Aberdeen) diluted in 2% BSA in PBS and incubate on ice for 1h. Cells were washed thrice in PBS and stained on ice for 45 min in secondary antibody (Invitrogen, goat anti-human IgG Fc, conjugated to Alexa Flour 488) diluted 1:200 in 2% BSA in PBS.

To stain for exposed chitin, 2.5 x 10^6 PFA fixed *Candida* cells were incubated on ice with 50 μg/ml TRITC-conjugated wheat germ agglutinin (Molecular Probes, Life Technologies) for 30 min. Cells were washed three times and suspended in PBS for analysis using Attune flow cytometry machine. Quantification of Aniline Blue (total glucan) and CFW (total chitin) fluorescence intensities were performed with the 405 nm excitation laser and emission filter set at 405 nm and 603/48 nm, whereas the FITC labelled cells were quantified using an excitation laser of 488 nm and emission filter of 530/30 nm. TRITC fluorescence labelled cells were quantified using the 488 nm excitation laser and 574/26 nm emission filter.Median fluorescence was determined from 10,000 events and corrected for background fluorescence.

2.7 Complement binding

2.7.1 Factor H binding

C. albicans cells were grown for 4 h in YPD broth with or without oestrogen 37ºC, 200 rpm. Cells were harvested and washed three times in PBS. To analyse Factor H binding, 2 x 10^6 yeast cells were incubated in 400 µL 10% normal AB human serum for 20 min at room temperature with slow shaking on a rotor. Cells were washed thrice in PBS, fixed on ice for 30 min with 4% PFA and blocked in 100 µL 2% BSA in PBS on ice for 30 min. Yeast cells were spun down, supernatant discarded and incubated on ice with 100 µL of 10 µg/mL Anti-Factor H Goat pAb (Sigma, 341276-1ML) diluted in 1% BSA in PBS. Cells were washed thrice in PBS and incubated in dark in 100 µL of Rabbit anti Goat IgG (H+L) Secondary Antibody, Alexa Fluor 488 (Invitrogen, A11078) diluted 1:200 in 1% BSA in PBS. Cells were washed thrice and analysed by Attune flow cytometry machine with excitation laser and emission filter set at 488 nm and 530/30 nm, respectively. Median fluorescence was determined from 10,000 events and corrected for background fluorescence.

2.7.2 C3 binding

C. albicans cells were grown for 4 h in YPD broth with or without 17β-estradiol at 37ºC, 200 rpm. About 1 mL of cells were harvested, washed thrice in PBS, and incubated with 400 µL 10% normal AB human serum for 20 min at room temperature with slow shaking on a rotor. Cells were blocked in 100 µL 2% BSA in PBS on ice for 30 min.

Candida cells were spun down, supernatant discarded and incubated on ice in 100 µL of 1 µg/mL Anti-Complement C3 Antibody (Sigma, AB3421) diluted in 1% BSA in PBS. Cells were washed three times in PBS and incubated in dark in 100 µL of Goat anti Chicken IgY (H+L) Secondary Antibody, Alexa Fluor 594, (Invitrogen, A11042) diluted 1:200 in 1% BSA in PBS. Cells were washed thrice and analysed by Attune FACS machine with excitation laser and emission filter set at 561 nm and 615/20 nm, respectively. Median fluorescence was determined from 10,000 events and corrected for background fluorescence.

2.7.3 C3b binding

C. albicans cells were grown for 4 h in YPD broth with or without 17β-estradiol at 37ºC, 200 rpm. About 1 mL of cells were harvested, washed thrice in PBS, and labelled with 400 µL 10% normal AB human serum for 20 min at room temperature with slow shaking on a rotor. Cells were blocked in 100 µL 2% BSA in PBS on ice for 30 min. Cells were spun down, supernatant discarded and incubated on ice in 100 µL of 1 µg/mL Anti-Complement C3b Antibody (Sigma, MABF982) diluted in 1% BSA in PBS. Cells were washed three times in PBS and incubated in dark in 100 µL of F(ab')2-Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 647 (Invitrogen, A-21237) diluted 1:200 in 1% BSA in PBS. Cells were washed thrice and analysed by Attune FACS machine with excitation laser and emission filter set at the 638 nm and 670/14 nm, respectively. Median fluorescence was determined from 10,000 events.

2.8 Molecular biology methods

2.8.1 Polymerase chain reaction (PCR)

All primers used for PCR are shown in Table 2.2. For general PCR, 1 µL of a desired concentration of DNA or RNA was added to the master mix prepared as shown in Table 2.3 below. While for colony PCR, a colony was picked with a pipette tip and emulsified in 50 µL RNase free water and 1 µL of the preparation was added to the PCR reaction mix. PCR was run following the set up as shown in Table 2.4. PCR products were run on 1 % agarose gel electrophoresis and imaged for the presence of required DNA fragment by UV transillumination with Bio-Rad ChemiDocTM MP imaging system.

2.8.2 Reverse transcriptase polymerase chain reaction

C. albicans cells from overnight culture were diluted into fresh YPD broth at 1:100 and grown for 4 h at 37 °C, 200 rpm in YPD broth with or without 10 µM 17β-estradiol. The qRT-qPCR was performed using 2x qPRBIO SyGreen mix kit (PCRbiosystem, PB25.11-12) according to manufacturer's recommendations with 50 ng of total RNA. *ACT1* was utilised as an endogenous control. The qRT-PCR machine set up conditions and primers used in the assay are shown in Table 2.5. Briefly, a master mix for each gene being tested was prepared by mixing 10 µL 2x qPCRBIO mix, 0.8 µL forward primer, 0.8 µL reverse primer and 1 µL RTase. The volume of the master mix was dependent on the number of samples to be tested.

12.6 µL of the mix was added into each well of the column assigned for each gene to be analysed. 7.4 µL of control and test RNA samples were added to their respective rows to make up a total volume sample to 20 µL per well. Each sample was analysed in duplicate. Ct values for control and test samples were normalised to actin Ct values as a reference gene. Relative quantification of gene expression was determined by using the formula $2^{-\Delta\Delta Ct}$ where Delta Delta Ct = $\Delta Ct_{\text{sample1}} - \Delta Ct_{\text{calibrator.}}$ Relative quantification is the fold change in comparison to the calibrator (untreated sample). For instance, a relative quantification of 10 would mean the gene is 10 times more expressed in the sample than in the calibrator whereas a relative quantification of 0.1 would mean the gene is 10 times less expressed.

Table 2.2. Primers used in this study

Primer	Sequence
GPD2-SacI-F	CTCCGAGCTCGGTGATGGTGATGGTGATGG
GPD2-NotI-R	GGAGAGCGGCCGCTGGTAAATTGGACAACGAGTG
EBP1-5F:	CTCCGATATCATCGCATGAG
EBP1-5R:	GGAGAGGAGCTCctgatgatatgataatttgc
EBP1-3F:	GGAGAGAAGCTTGGGAATGAAGTTCTATTAGC
EBP1-3R:	GGAGAGGGTACCgttacatctactactacagg
GPD2-OE-F	**GGAGAG**ccgcggATGACTACTTCCCCATATCCAAT
GPD2-OE-R	**GGAGAG**gcggccgcACGCAGAGAACAAGAACGTC
RT-ACT1-F	CCTACGTGTACTTGTGCAAGGCAA
RT-ACT1-R	TAGTTGTGTGCACTGAGCGTCGAA
RT-GDP2-F	GCCAACGAAGTTGCCAAAGGT
RT-GPD2-R	AGGCACCAGCAATAGAGGCA

Table 2.3. PCR reaction mix

Component	Volume
5X Phusion HF buffer	10 µL
10 mM dNTPs	1.0 µL
10 µM Forward primer	1.0 µL
10 µM Reverse primer	1.0 µL
Candida cells/100 ng Template DNA	1.0 µL
Phusion DNA polymerase	0.5 µL
Nuclease free water	35.5 µL
Total volume	50 µL

Table 2.4. General PCR settings

Cycles	Temperature	Time	Stage
1	95°C	5-10 min	Initial denaturation
	95°C	30 s	Denaturation
35	55°C	30 s	Primer annealing
	72°C	1 min	Extension
1	72°C	5 min	Final Extension
	10°C	∞	Hold

Table 2.5. RT-qPCR settings

Cycles	Temperature	Time	Stage
1	45°C	10 min	Reverse transcription
1	95°C	2min	Polymerase activation
40	95°C	5 seconds	Denaturation
	59°C	30 seconds	Anneal/Extension

2.8.3 Agarose gel electrophoresis

To separate DNA/RNA, 1 % agarose gel (MP Biomedicals, 820723) in 50 mL 1 X TBE buffer (10 mM TRIS, 150 NaCL at pH 7.6) was dissolved by boiling. The gel was cooled down to 50 ^{o}C and 2 µL SYBR safe DNA gel stain (Fisher Scientific, S33102) was added.

The gel was poured into casting tray and a comb was put in the cast to generate sample loading wells. After 30 min, the gel cast was placed in electrophoresis tank and 5 µL DNA/RNA samples were mixed with 1 µL loading dye (NEB, B7024S) and loaded onto respective wells. Samples were separated for 35 min with current set at 80 volts. Gene ruler 1Kb Plus DNA ladder (ThermoFisher-scientific, SM1331) was also run together with the samples to help in estimating the molecular weight of separated nucleic acid samples. DNA/RNA fragments were detected by UV transillumination using Bio-Rad ChemiDocTM MP imaging system.

2.8.4 RNA extraction

RNA extraction was performed as previously described (Sherrington *et al.,* 2017). *C. albicans* cells from overnight culture were diluted into fresh YPD broth at 1:100 and grown for 4 h at 37 °C, 200 rpm in YPD broth with or without 10 µM 17β-estradiol. Cells were harvested by centrifugation at 3500 rpm for 5 min. Pellet was re-suspend in 200 µL YPD broth media. Cells were slowly added by drops to a 50 mL falcon tube filled 25 % with liquid nitrogen. Small droplets of snap frozen samples were transferred to RNAse free 1.5 mL Eppendorf tube and kept at -80 °C until ready to extract RNA. Samples were retrieved from -80 °C freezer and 600 µL RLT1 buffer supplemented with 10 µL beta-mercaptoethanol was added to the tubes. Samples were defrosted on ice. Samples were transferred to screw capped tubes containing glass beads and bead beaten for five cycles (2 x 20 s @ 6500 rpm) with 5 min on ice between each cycle. Cell lysis was confirmed under a microscope.

Samples were transferred to QIA shredder column and centrifuged for 2 min at 13,000 rpm. Flow through was transferred to gDNA eliminator column and centrifuged at 10,000 rpm for 1 min. Next, 350 µL of 70% ethanol was added to the supernatant and samples were transferred to RNeasy column (pink). Samples were centrifuged at 10,000 rpm for 30 s. Flow through was discarded and 350 µL RW1 buffer was added, and samples were centrifuged at 10,000 rpm for 30 s. 70 µL RDD buffer was added to 10 µL of RNAse free DNAse, mixed and added to centre of column. Samples were incubated at room temperature for 20 min. 350 µL RW1 buffer was added and centrifuged at 10,000 rpm for 30 s.

Flow through was discarded and 500 µL RPE buffer was added, and samples were centrifuged at 10,000 rpm for 30 s. Flow through was discarded and 500 µL RPE buffer was added, and samples were centrifuged at 10,000 rpm for 2 min. Flow through was discarded and columns were transferred to fresh collection tubes and centrifuged at 13,000 rpm for 1 min. Column was transferred to fresh 1.5 mL Eppendorf tube and 50 µL RNase free water was added. Column was incubated at room temperature for 1 min and centrifuged at 10,000 rpm for 1 min. Another 30 µL RNase free water was added to the column and incubated at room temperature for 1 min and then centrifuged at 10,000 rpm for 1 min. RNA concentration was determined using a NanoDrop. Genomic DNA contamination was checked by PCR and RNA quality was checked by agarose gel electrophoresis.

2.8.5 RNA sequencing

RNA samples were extracted and prepared as described above. Samples sent for RNA sequencing were processed as described by Cottier *et al*., (2019). Using the accession number GSE145240, sequencing reads can be found at the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/). CLC Genomic workbench 11.0.1 (Qiagen) was utilised to analyse the sequencing reads using a previously published protocol (Cottier *et al.,* 2019). Analysis of RNA sequencing data was performed by Dr Fabien Cottier, a post-doctoral research fellow in our research group. Raw FASTQ sequence reads were formatted and imported into CLC Genomic workbench 11.0.1 software (Qiagen) for analysis.

Briefly, adapter and quality trimming of the reads was carried out prior to mapping to *C. albicans* reference genome (Assembly 21, version s02-m09-r10). A report on Transcript Per Kilobase Million (TPM) was generated for each open reading frames (ORFs). The values of smallest TPM measurements were added and statistical analysis was done. This was followed by log10 transformation of the data. Genes were considered significantly differentially expressed if the absolute value of Fold Change was >2 and the FDR was < 0.001. Gene ontology (GO) analysis with P-values corresponding to Bonferroni-corrected hypergeometric test P-values was performed using CGD GO term finder (Inglis *et al.,* 2012).

2.8.6 Genetic manipulation of *C. albicans*

2.8.6.1 Generating of GPD2 reintegrate strain

To reintroduce *GPD2* into *C. albicans gpd2*Δ, the *GPD2* locus was amplified from *C. albicans* SC5314 genomic DNA by PCR using the primers GPD2-SacI-F and GPD2-NotI-R. The PCR product and CIp30 plasmid were restricted with SacI (Thermo Scientific, FD1134) and NotI (Thermo Scientific, FD0594). The restriction products were dephosphorylated and purified as described below. The DNA fragment was cloned into CIp30 plasmid to generate CIp30-*GPD2*. Cloning was done at a vector to Insert molar ratio of 1:3 using T4 DNA ligase (Thermo Scientific, EL0011); (1 µL T4 DNA ligase, 2 µL ligation buffer, vector DNA, insert DNA and RNase free water up to 20 µL then incubate overnight at 4°C).

CIp30-*GPD2* was linearized with StuI (New England Biolabs, R0187S) and transformed into *C. albicans gpd2*Δ using standard heat-shock transformation to generate the *GPD2*-reintegrant strain (Walther *et al.,* 2003). The integration of CIp30-*GPD2* in *C. albicans* gpd2 mutant was confirmed by PCR.

2.8.6.2 Generating of *GPD2* overexpressing strain

To generate a *C. albicans* strain that over expresses *GPD2*, the open reading frame of *GPD2* was cloned into pSM2 using primers GPD2-OE-F and GPD2-OE-R under the control of the *TEF2* promoter. The resulting plasmid was linearized with PacI and integrated into CAI4 at the *URA3* locus by standard heat-shock transformation.

2.8.7 *E. coli* heat shock transformation

E. coli heat shock transformation was carried out as previously described (Froger *et al.,* 2007). Chemically competent *E. coli* cells (50 µL) were defrosted on ice and then 20 µL of the ligation reaction was added. Cells were gently mixed by tapping side of tube. Cells were incubated on ice for 30 min and then heat-shocked at 42ºC for 1 min. Next, 600 µL of LB broth medium was added to the tube and incubated at 37ºC, 200 rpm for 45 min. Sample was centrifuged at 5000 rpm for 2 min. Supernatant was discarded and cells were suspended in 100 µL of LB broth. Sample was spread on LB agar containing 100 µg/mL ampicillin. Plates were dried and incubated at 37 ºC for 18 hr. Colonies were picked and sub-cultured onto fresh plates to confirm resistance. Colony PCR was performed to check for successful transformation.

2.8.8 Extraction and purification of plasmid DNA

Miniprep purification of plasmid DNA was carried out as per manufacturer's instructions. QIAprep Spin Miniprep Kit (Qiagen, 21704VF) was used for purifying plasmid DNA. About 5 mL the *E. coli* DHα overnight culture was centrifuged at 13,000 rpm for 3 min at room temperature. Pellet was re-suspended in 250 µL Buffer P1 and transferred to a 1.5 mL microcentrifuge tube. Buffer P2 (250 µL) was added to the tube and thoroughly mixed by inverting until the solution was clear. This was done for no more than 5 min and then 250 µL Buffer N3 was added and immediately mixed by inverting the tube until the mixture became colourless.

The tube was centrifuged for 10 min at 13,000 rpm. The supernatant was pipetted to the QIAprep spin column and centrifuged for 1 min. The flow through was discarded and the QIAprep spin column was washed with 500 µL Buffer PB by centrifuging for 1 min at 13,000 rpm. The flow through was discarded and 750 µL Buffer PE was added for centrifuging at 13,000 rpm for 1 min. Flow through was discarded and the QIAprep spin column was further spun for 1 min to remove residual wash buffer. The QIAprep column was placed in a clean 1.5 mL Eppendorf tube. To elute plasmid DNA, 50 µL of RNase free water was added to the centre of the QIAprep column, left to stand for 1 min and centrifuged for 1 min. Sample was analysed for DNA concentration using a NanoDrop.

2.8.9 Dephosphorylation of restriction digest products

Dephosphorylation of restriction digest products was performed as per manufacturer's instructions. Briefly, plasmid DNA/linear DNA (1 µg) was mixed with 2 µL 10X Thermo Scientific FastDigest Buffer (Thermo Fisher scientific), 1 µL FastDigest Restriction Enzyme (Thermo Fisher scientific), 1 µL FastAP Thermosensitive Alkaline Phosphatase (Thermo Fisher scientific) and top up to 20 µL with RNAse free water. Preparation was mixed thoroughly and incubated overnight at 37 °C.

2.8.10 Purification of restriction digest and dephosphorylation products

Purification of restriction digest and dephosphorylation products was performed as per manufacturer's instructions. QIAquick PCR Purification Kit (Qiagen, 128104VF) for the assay. Five volumes of Buffer PB were added to one volume of the products. A QIAquick column was placed in a 2 mL collection tube. To bind DNA, samples were applied to the QIAquick column and centrifuged for 1 min. Flow through was discarded and the QIAquick column was placed back in the same tube. The QIAquick column was washed with 750 µL Buffer PE by centrifuging for 1 min. Flow through was discarded and the QIAquick column was put back into the same tube. To remove residual wash buffer, the QIAquick column was placed in a fresh 2 mL collection tube and centrifuged for 1 min. The QIAquick column was placed in a clean 1.5 mL Eppendorf tube. DNA was eluted by adding 50 µL RNase water to the centre of the QIAquick membrane and incubated for 1 min before centrifuging for 1 min. Another 30 µL RNase water was added to the centre of the QIAquick membrane and the column was incubated for 1 min and then centrifuged for 1 min. The product was analysed by agarose gel electrophoresis to check for the DNA.

2.9 *In vivo* experiments

2.9.1 *Galleria mellonella* larva infection

Overnight *C. albicans* culture was diluted 1:100 and cultured for 4 h in YPD broth at 37 °C, 200 rpm with test *Candida* cultures supplemented with 10 μM and 0.001 μM 17β-estradiol, respectively. Cells were harvested by centrifugation, washed three times in PBS and re-suspend in PBS at 10^6 per 20 μL. Using an insulin syringe, larvae were inoculated with 20 μL of *Candida* suspension by injecting through the last left proleg into the haemocoel. Larvae were incubated at 30°C in the dark for up to 96 hrs. Controls included, non-infected larvae, mock infected larvae, 17β-estradiol primed larvae and larvae infected with wild type *Candida*. Two hours prior to infection, the mock infected larvae, and larvae to be inoculated with *Candida* control were injected with 20 μL PBS while test larvae were primed with 20 μL 17β-estradiol (10 μM and 0.001 μM respectively). Except for non-infected control, all other controls were injected with 20 μL of PBS injected just before infection. This was to ensure that the total volume of PBS injected is the same for all larvae as well as to make sure that all larvae are subjected to the same level of injection trauma. Each experimental condition had ten healthy larvae placed in a sterile 9-cm Petri dish with a filter paper. Larvae were assessed every 12 hrs for viability and disease progression. To assess viability, larvae were gently probed with a needle, and if no response was observed, the larvae was considered dead.

Changes in cuticle melanisation were also monitored to assess severity of infection. Data from three independent experiments were pooled together for analysis to determine the survival rate of the larvae.

2.9.2 Zebrafish larvae infection

Wild type (AB) Danio rerio zebrafish used in the study were housed in a recirculating system of gallon tanks at the University of Birmingham Zebrafish Facility. To obtain embryos, 4 male and 5 female fish were transferred into a breeding tank and maintained at 28 °C, 14 h light/10 hr dark cycle. Embryos were collected the following day, sorted, and incubated at 30 °C for 24 hr in E3 media (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2, 0.33 mM MgSO4, 0.00003% methylene blue, pH 7). Embryos were maintained at a density of 100 per 14.5 cm dish containing 150 mL E3 media supplemented with 0.02 mg/mL Phenylthiourea. *C. albicans* cells from overnight culture were diluted 1:100 and sub-cultured for 4 hr in YPD broth at 37°C, 200 rpm. Cells were harvested by centrifugation, washed three times in PBS and re-suspend at 5×10^7 cells/mL in injection buffer (10% PVP-40 in PBS, 0.05% phenol red).

With few modifications, hind brain infections were performed as previously described (Mallick *et al.,* 2016). Briefly, day zero post fertilization (dpf), about 100 good embryos were sorted into a 14.5 cm petri dish with 150 mL 1x E3 media. Test embryos were placed in media supplemented with 1 µM or 10 µM 17β-estradiol. Eggs were incubated at 28 °C in E3 media until they reach the prim25 stage the following day.

Zebrafish at the prim25 stage were manually dechorionated, and transferred into a 9 cm petri dish with 25 mL E3 supplemented with anaesthesia (160 µg/mL Tricaine) for at least 10 min. Following calibration of microinjection needles, fish were transferred onto 2 % agar plate and microinjected with approximately 5 nL of injection buffer or *C. albicans* suspension at 5×10^7 cells/mL in injection buffer into the hindbrain ventricle via the otic vesicle to achieve a dose of 20-50 yeast/larva. Zebrafish larvae were transferred into another 9 cm petri dish with 25 mL E3 for recovery. Within 1 hr of infection, larvae were screened by microscopy to remove fish noticeably traumatised from microinjection and to ascertain correct injection site and inoculum size. At least 15 larvae per condition were transferred into a 6-well plate, incubated at 28 °C in E3 media and observed for survival every 24 hr until day 5 post fertilisation. Depending on the experiment, some zebrafish were incubated in E3 media supplemented with 1 µM or 10 µM 17β-estradiol. Data from three independent biological replicates were pooled together to determine percent survival.

2.10 Statistics

Unless indicated otherwise, data were analysed in GraphPad Prism (version 7) using a Kruskal-Wallis test followed with a post-hoc Dunn's multiple comparisons test at 95 % confidence. Data represent the mean +/- SEM from at least three independent experiments. To test if values came from a Gaussian distribution a D'Agostino & Pearson normality test was performed.

In addition, data for survival experiments for the zebrafish and *Galleria mellonella* larvae from at least three independent experiments was pooled together and significant differences between the test and control were determined by Log-rank (Mantel-Cox test).

3. Characterisation of the effects of oestrogen on *Candida albicans*

3.1 Background

A wide variety of studies on oestrogen and its role in regulating innate immune system have been extensively conducted in mammals. Oestrogen can influence the role of innate immune cells to render the host susceptible or resistant to infections. For instance, treatment of U937 macrophages by oestrogen enhances the production of TNF-α and reduces the synthesis of IL-10 thereby promoting inflammation (Carruba *et al.,* 2003). On the other hand, oestrogen displays anti-inflammatory effect on murine monocytes by suppressing the expression of chemokine receptors CCR2 and CXR3 and also by inhibiting their chemotaxis towards Macrophage Inflammatory Protein-1α (MIP-1α) and Monocyte Chemoattractant Protein-1 (MCP-1) (Janis *et al.,* 2004). Similarly, oestrogen delays the recruitment of neutrophils in vagina by regressing the gradient of Cxcl1, a ligand for neutrophil chemokine receptor Cxcr2 (Lasarte *et al.,* 2016). This renders the host susceptible to vaginal candidiasis.

Oestrogen is a key predisposing factor to VVC but there is limited knowledge regarding the direct effects of the hormone on the fungus that could influence its virulence. Nevertheless, other host niches are reported to enhance the ability of the fungus to cause infection. For instance, exposure of *C. albicans* to hypoxic conditions leads to modulation of glucan exposure and subsequent reduction in neutrophil phagocytosis (Lopes *et al.,* 2018).

As such, the main objective of this chapter was to investigate whether *C. albicans* can adapt to oestrogen in a way that promotes its own virulence. Thus, the investigation focused on determining the impact of the hormone on growth and morphology of *C. albicans*, and how *C. albicans* adaptation to oestrogen affects phagocytosis and secretion of cytokines by innate immune cells.

3.2 Results

3.2.1 Oestrogen does not affect the morphology or growth rate of *C. albicans* when grown in YPD.

Morphogenesis is an important attribute in the pathogenesis of *C. albicans*. Transition from one morphological state to the other happens in response to modifications in the growth environment (Nadeem *et al.,* 2013). To explore the impact of oestrogen on morphology of *C. albicans*, cells were grown in YPD broth supplemented with oestrogen and examined for changes in size and shape. Results indicated that exposure of the fungus to oestrogen does not cause significant changes in size (Figure 3.1A). Similarly, the shape of *C. albicans* was not significantly affected by oestrogen treatment (Figure 3.1B-C).

In the host, *C. albicans* is exposed to a repertoire of environmental cues that are both favourable as well as hostile for its growth and survival (Braunsdorf *et al.,* 2016). To assess whether adaptation to oestrogen influences the growth rate of the fungus, *C. albicans* cells were grown in YPD broth complemented with oestrogen and incubated for 18 h in a plate reader.

Oestrogen did not affect the growth rate of *C. albicans* (Figure 3.2A-I), with the average doubling time in the control and oestrogen containing media being 2.6 h. To confirm that other physiological forms of oestrogen do not affect the growth of *C. albicans* the growth of *C. albicans* is the presence of 17β-estradiol, 17α-ethynylestradiol and estriol was determined. Neither form of oestrogen had a significant effect on the growth rate of *C. albicans* (Figure 3.2A-I).

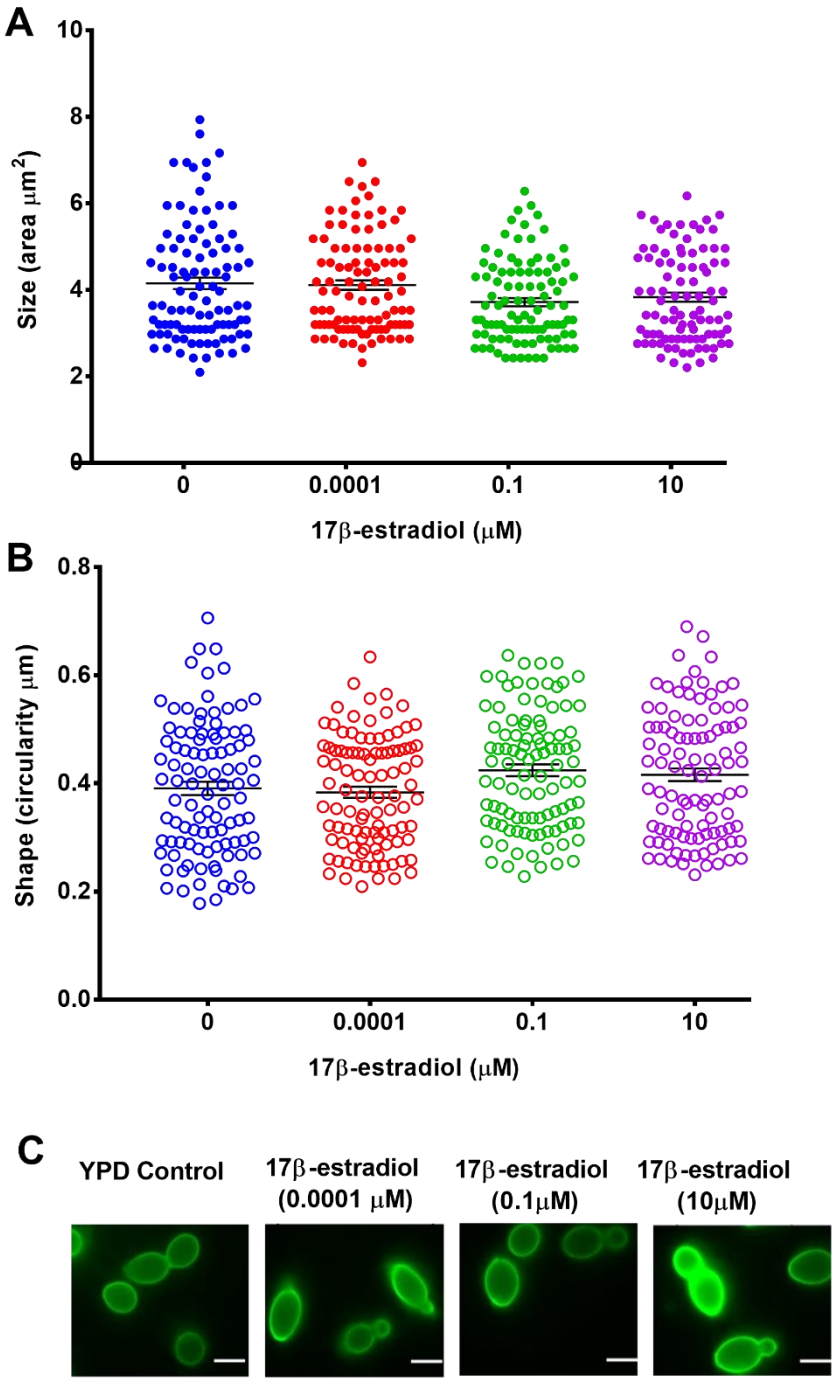

Figure 3.1. Oestrogen does not affect morphology of C. albicans in YPD. *C. albicans* cells were grown for 4 h at 37°C, 200 rpm in YPD broth supplemented with different concentrations of oestrogen. Cells were harvested, washed with PBS, and imaged by microscopy. One hundred cells per condition were analysed for changes in (**A**) cell size and (**B**) shape using ImageJ software. (**C**) Images of *C. albicans* cells post oestrogen treatment.

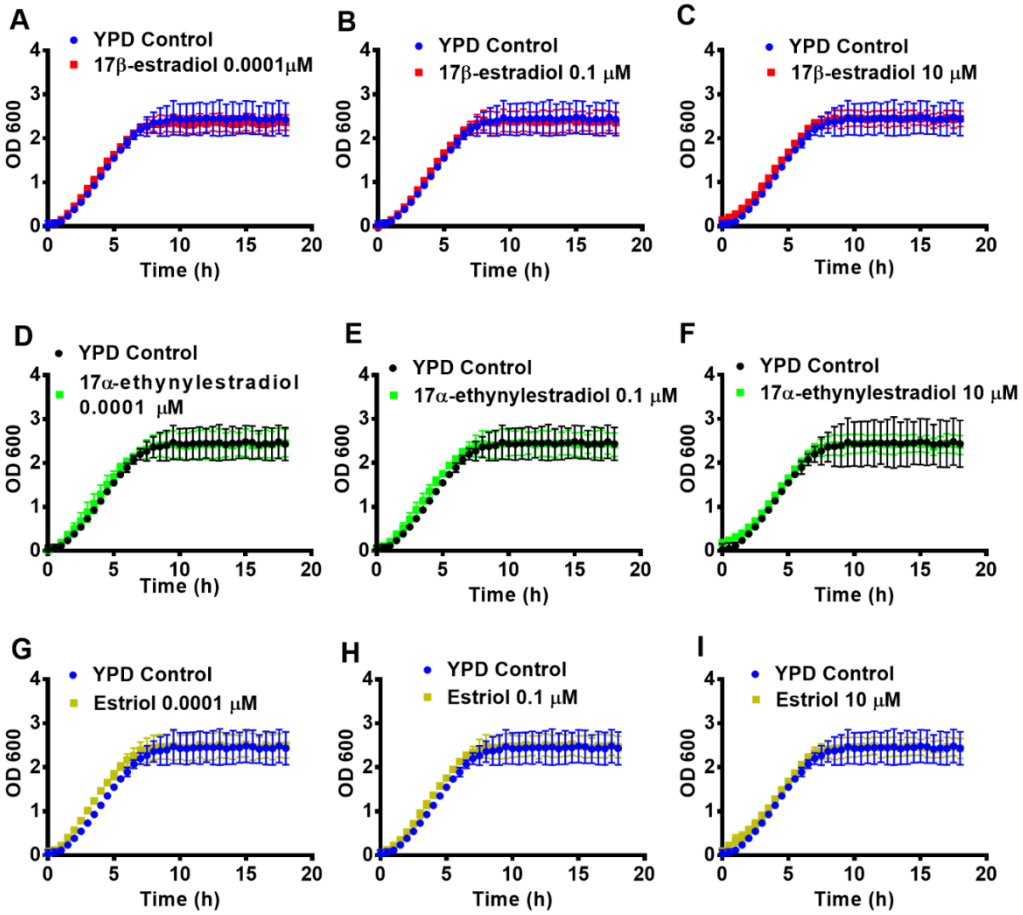

Figure 3.2. Oestrogen does not influence the growth of *C. albicans* in YPD broth. YPD broth cultures of *C. albicans* cells supplemented with varying concentrations of oestrogen were inoculated in a 96 well plate and set in a plate reader for 18 h at 37°C. Optical densities (OD) of the cultures were recorded every 30 min and values were utilised to determine growth curves for 17β-estradriol (**A**, **B**, **C**), 17α-Ethynylestradiol (**D**, **E**, **F**) and Estriol (**G**, **H**, **I**). All data represent the mean ± SEM from two independent experiments done in duplicates.

3.2.2 Adaptation of *C. albicans* to oestrogen promotes immune evasion.

Women taking oestrogen-containing oral contraceptives are more prone to vulvovaginal candidiasis (VVC) (Neerja *et al.,* 2006, Cetin *et al.,* 2007, Oviasogie *et al.,* 2009, Egbe *et al.,* 2011, Apalata *et al.,* 2014).

This suggests that oestrogen promotes the virulence of *C. albicans*. To ascertain whether adaptation of *C. albicans* to oestrogen affects the host-pathogen interaction, *C. albicans* cells were grown in the presence of physiological (0.0001 µM) and super-physiological (0.1 µM, 10 µM) concentrations of oestrogen and then co-incubated with J774A.1 macrophages to analyse phagocytosis rates. Physiological and super-physiological concentrations of oestrogen significantly inhibited phagocytosis of *C. albicans* by macrophages (Figure 3.3A-C). This effect was observed with adaptation of *C. albicans* to 17β-estradiol (Figure 3.3A), estriol (Figure 3.3B), and 17α-ethinylestradiol (Figure 3.3C). Since all tested forms of oestrogen elicited similar results, all subsequent experiments were performed using 17β-estradiol.

Following the observation that oestrogen-adapted *Candida* cells attenuated phagocytosis by J774A.1 cells, an examination was carried out to verify whether this phenotype could be exhibited with human derived phagocytes. 17β-estradiol-adapted *C. albicans* cells were incubated with human monocyte-derived macrophages (HMDM) and neutrophils and phagocytosis quantified.

There was a significant drop in phagocytosis of 17β-estradiol-adapted *Candida* cells by both HMDM (Figure 3.4A) and neutrophils (Figure 3.4B), confirming that this effect was not generating from the use of the macrophage cell line.

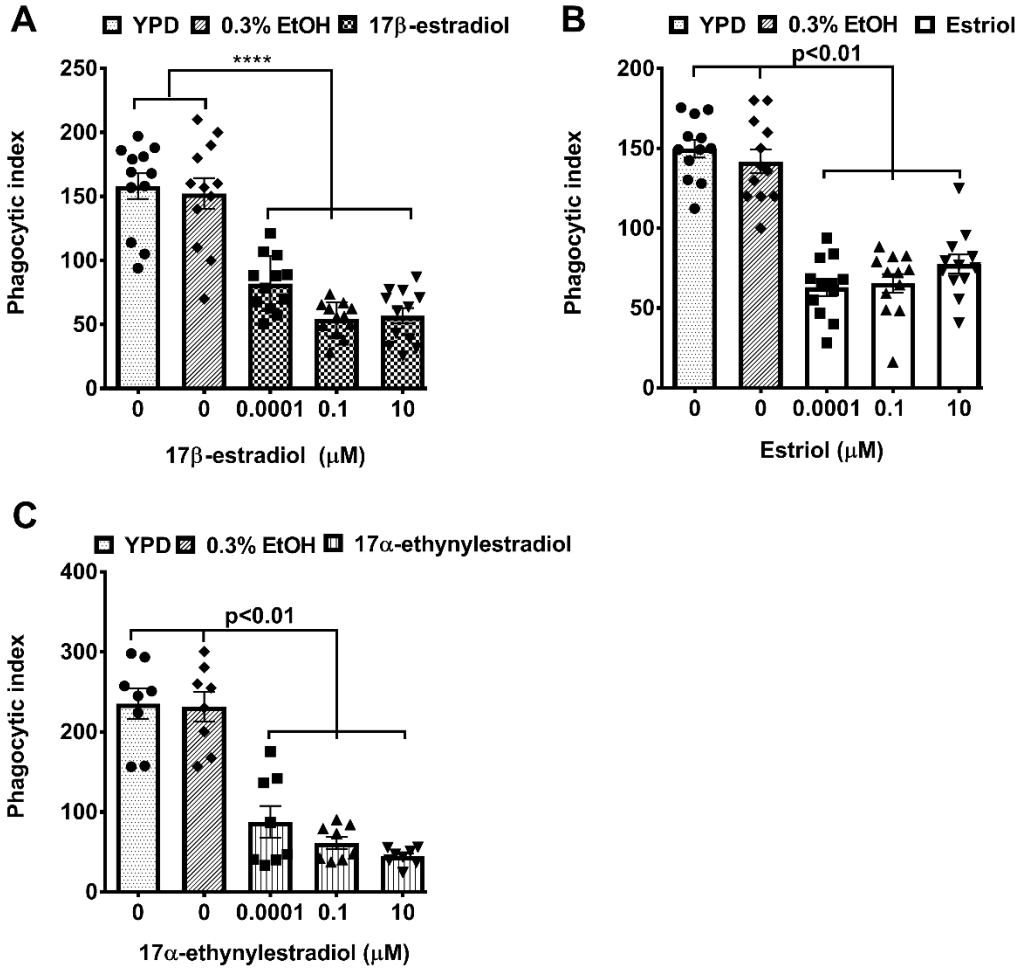

Figure 3.3. Oestrogen promotes innate immune evasion of *C. albicans*. *C. albicans* cells (SC5314) were grown for 4 h in YPD with or without oestrogen supplementation. Cells were harvested, washed in PBS and co-incubated with J774A.1 cells, MOI = 5. (**A**) 17β-estradiol (**B**) estriol and (**C**) 17α-ethynylestradiol. All data represent the mean ± SEM from twelve biological replicates. Data were analysed by (**A**) ordinary One-way ANOVA test, (**B)** and (**C)** Kruskal-Wallis test and Tukey's multiple comparisons test; ****p<0.0001.

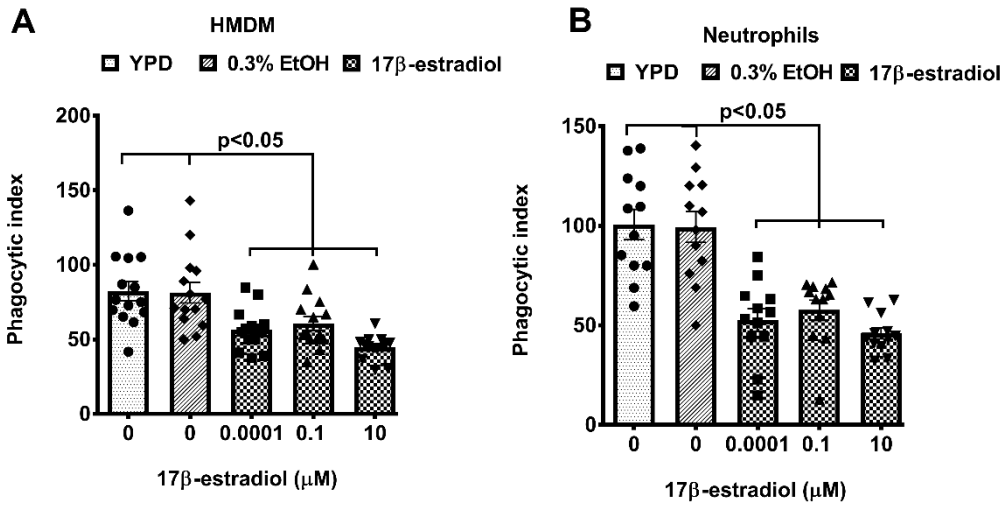

Figure 3.4. Innate immune evasion in response to oestrogen occurs in primary human innate immune cells. *C. albicans* cells were grown for 4 h in YPD with or without oestrogen supplementation. Cells were harvested, washed in PBS and co-incubated with (**A**) HMDM (**B**) neutrophils. Phagocytosis rates of *C. albicans* cells were determined. All data represent the mean ± SEM from at least twelve biological replicates. Data were analysed by (**A)** ordinary One-way ANOVA test, **B** Kruskal-Wallis test) and Tukey's multiple comparisons test.

3.2.3 *C. albicans* clinical isolates and laboratory strains respond to oestrogen in a similar manner.

To explore whether clinical *C. albicans* isolates and laboratory strain SC5314 exhibit a similar phenotype after adaptation to oestrogen, *C. albicans* vaginal isolates from asymptomatic (SVS063A) and symptomatic (SVS006B) clients were tested on how they would respond to oestrogen. Similar to *C. albicans* SC5314, all oestrogen-adapted vaginal isolates inhibited phagocytosis (Figures 3.5A-B). This was regardless of whether the isolates were retrieved from healthy women or VVC patients. Therefore, the oestrogen-induced immune evasion is a general trait of C*. albicans*.

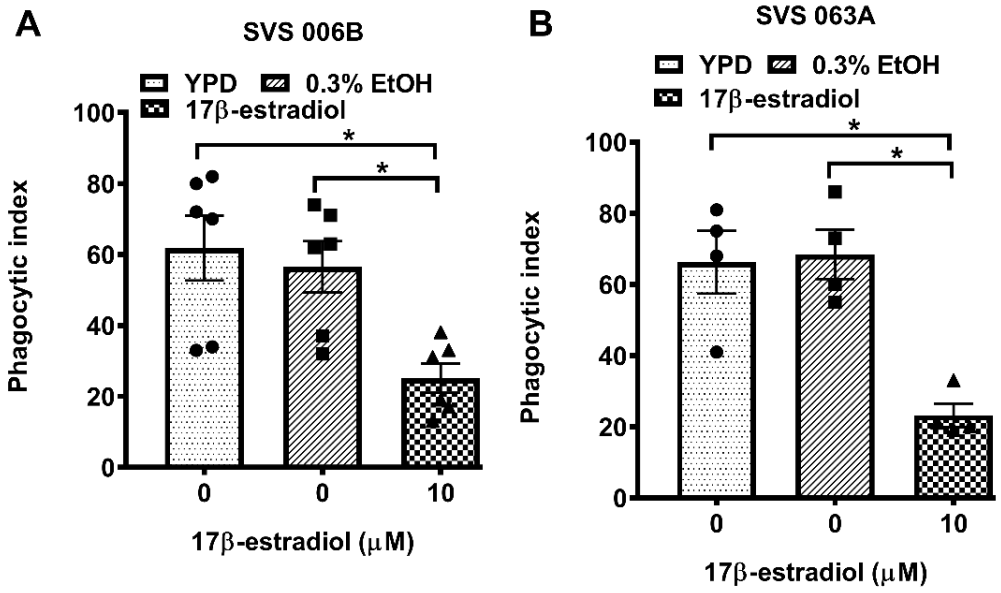

Figure 3.5. Oestrogen promotes innate immune evasion of *C. albicans* clinical isolates. *C. albicans* cells (**A**) SVS 006B and (**B**) SVS 063A were grown for 4 h in YPD with or without oestrogen supplementation. C. albicans cells were harvested, washed in PBS and co-incubated with J774A.1 cells. Phagocytosis of C. albicans cells was determined. All data represent the mean ± SEM from at least four independent experiments. Data were analysed by Kruskal-Wallis test and Dunn's multiple comparisons test; *p<0.05.

3.2.4 Response to oestrogen varies among *Candida* species.

After *C. albicans*, *C. glabrata* is the second most common cause of VVC (Gonçalves *et al.,* 2016). Other non-*albicans Candida* species have also been isolated from the female reproductive tract. Here, the ability of oestrogen to promote immune evasion in non-*albicans Candida* species was determined. *Candida* species analysed included *C. glabrata, C. dublinensis*, *C. parapsilosis* and *C. tropicalis*. Out of these species, only *Candida tropicalis* displayed reduced phagocytosis after adaptation to oestrogen (Figure 3.6A-D).

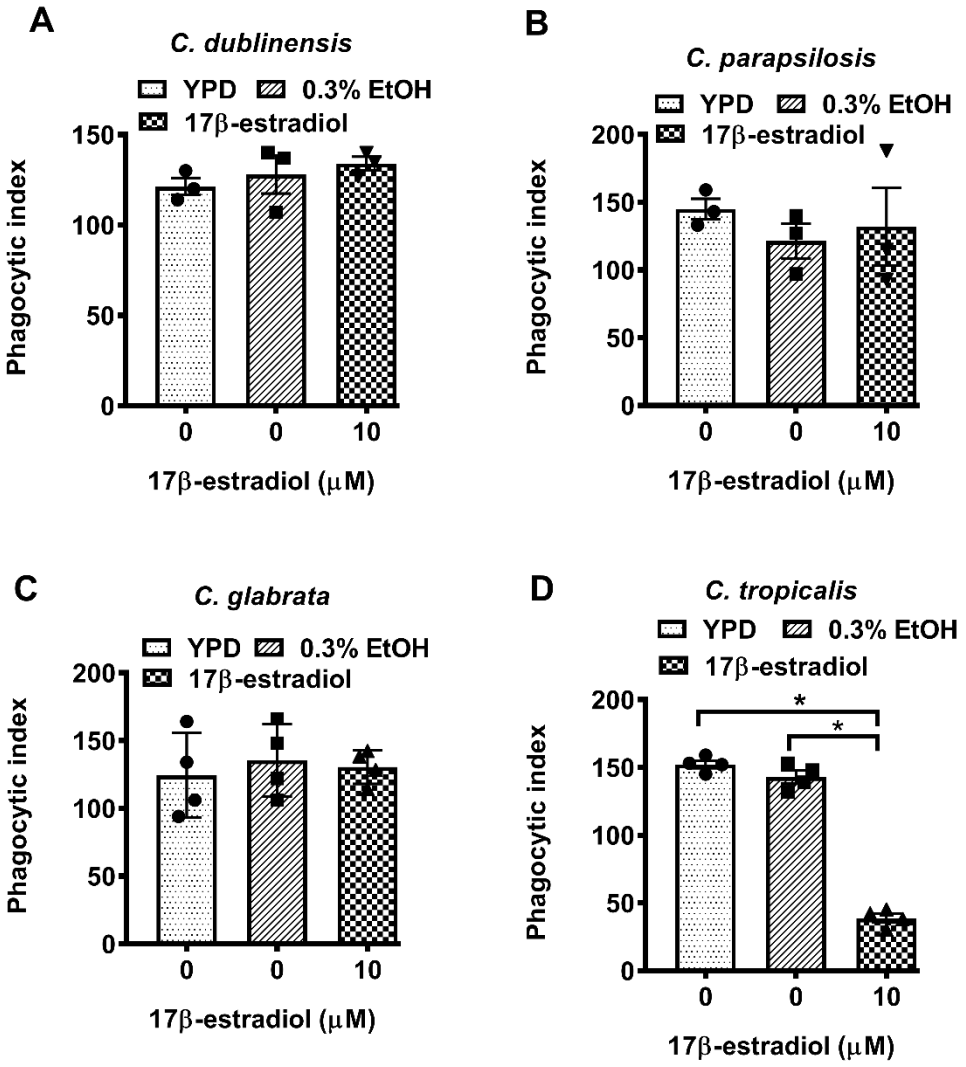

Figure 3.6. Oestrogen does not promote innate immune evasion in all *Candida* species. *Candida* cells were grown for 4 h in YPD with or without oestrogen supplementation, harvested, washed in PBS and co-incubated with J774.1A cells. Phagocytosis of *Candida* cells was quantified. (**A**) *C. dublinensis* (**B**) *C. parapsilosis* (**C**) *C. glabrata* and (**D**) *C. tropicalis* All data represent the mean ± SEM from at least three independent experiments. Data were analysed by Kruskal-Wallis test and Dunn's multiple comparisons test; *p<0.05.

In addition, the effect of oestrogen on *S. cerevisiae* host-pathogen interaction was also evaluated. Like *C. albicans* and *C. tropicalis*, oestrogen-adapted *S. cerevisiae* exhibited reduced phagocytosis (Figure 3.7). Therefore, the oestrogen-dependent innate immune evasion varies among *Candida* species, but it can be extended to other yeast organisms such as *S. cerevisiae*.

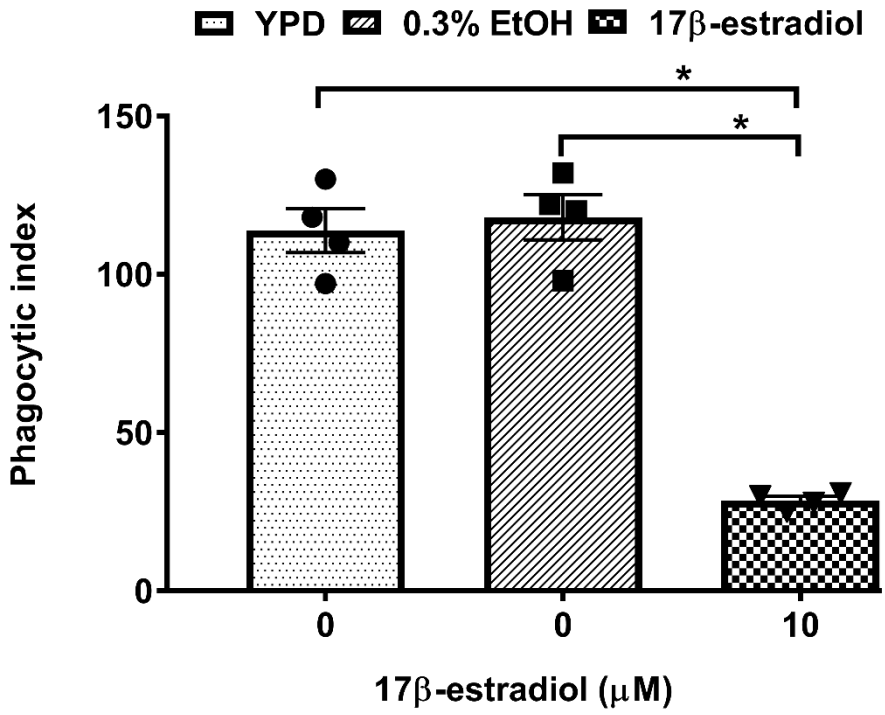

Figure 3.7. Oestrogen promotes innate immune evasion of *S. cerevisiae.* *S. cerevisiae* cells were grown for 4 h in YPD with or without oestrogen supplementation. Cells were harvested, washed in PBS and co-incubated with J774A.1 cells. Phagocytosis of yeast cells was quantified. All data represent the mean ± SEM from at least three independent experiments. Data were analysed by Kruskal-Wallis test and Dunn's multiple comparisons test; *p<0.05.

3.2.5 The oestrogen-induced immune evasion is attributable to the impact of the hormone on *C. albicans.*

Knowledge on effects of oestrogen on phagocyte function is still growing however studies show that the hormone does suppress the function of human phagocytes (Bodel *et al.,* 1972, Sivam *et al.,* 1987, Miyagi *et al.,* 1992, Lasarte *et al.,* 2016). Therefore, experiments were performed to establish whether the observed inhibition of phagocytosis is truly mediated by adaptation of *C. albicans* to oestrogen, and not a result of residual oestrogen coating the surface of *C. albicans* and inhibiting phagocyte function. Firstly, macrophages were pre-treated with oestrogen prior to incubation with *C. albicans* and latex beads, respectively.

It was noted that macrophages pre-incubated with oestrogen phagocytosed latex beads (Figure 3.8A) and *C. albicans* (Figure 3.8B) at rates like the control (i.e., non-oestrogen exposed macrophages). The outcome indicated that oestrogen does not influence the phagocytic ability of macrophages *in vitro* thus suggesting that the inhibition of phagocytosis observed was probably not because of residual oestrogen on *C. albicans* altering the phagocytic function of macrophages.

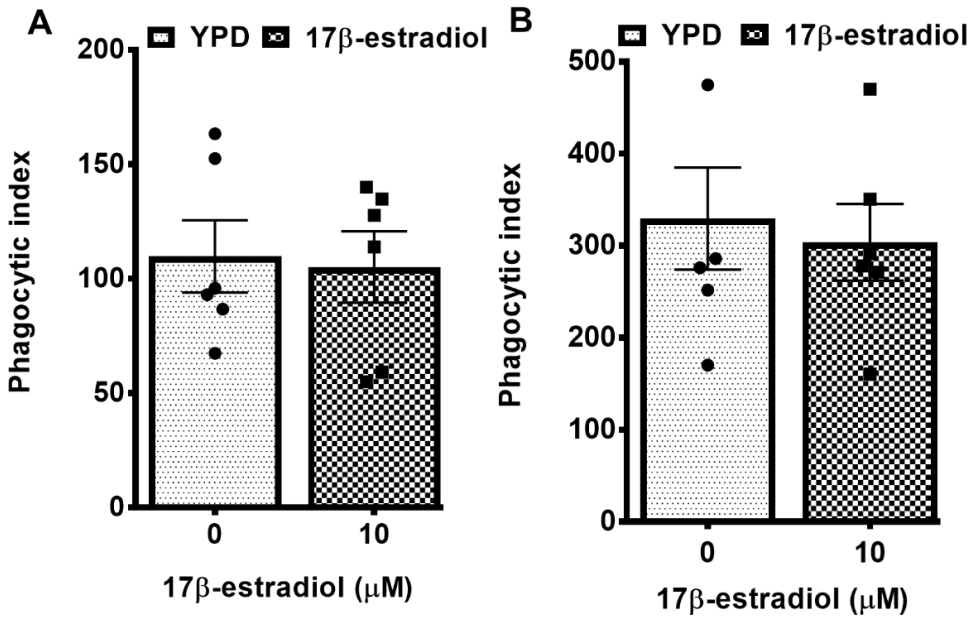

Figure 3.8. Oestrogen does not influence the phagocytic ability of J774A.1 macrophages. J774A.1 macrophages pre-treated with 10 μM 17β-oestradiol were incubated for 4 h with (**A**) beads and (**B**) wild type *C. albicans* SC5314 cells grown in YPD, and phagocytosis rates were quantified. All data represent the mean ± SEM from at least three independent experiments.

Next, inert particles were used to examine whether oestrogen could coat the particles and influence their uptake by macrophages. Latex beads were incubated in PBS supplemented with oestrogen and then washed for co-incubation with macrophages. Irrespective of oestrogen treatment, the phagocytosis rate of beads was the same in test and control conditions (Figure 3.9A). The expectation was to see a reduction in phagocytosis if the process of inhibiting uptake was based on impact of residual oestrogen on macrophages. Thus, this observation further supports the theory that oestrogen is inducing changes on *C. albicans* which then facilitates reduction in phagocytosis.

Furthermore, an investigation was conducted to find out whether the effect of *C. albicans* adaptation to oestrogen is permanent or temporary. *Candida* cells were exposed to oestrogen for 4h and then washed and re-incubated in fresh YPD broth for 24 h. Cells were washed and co-incubated with macrophages. Sub-culturing oestrogen-adapted *C. albicans* cells into fresh YPD media resulted into restoration of phagocytosis rates to that of control cells (Figure 3.9B). Altogether these data suggest that adaptation of *C. albicans* to oestrogen is a reversible process and is required to facilitate immune evasion of *C. albicans*.

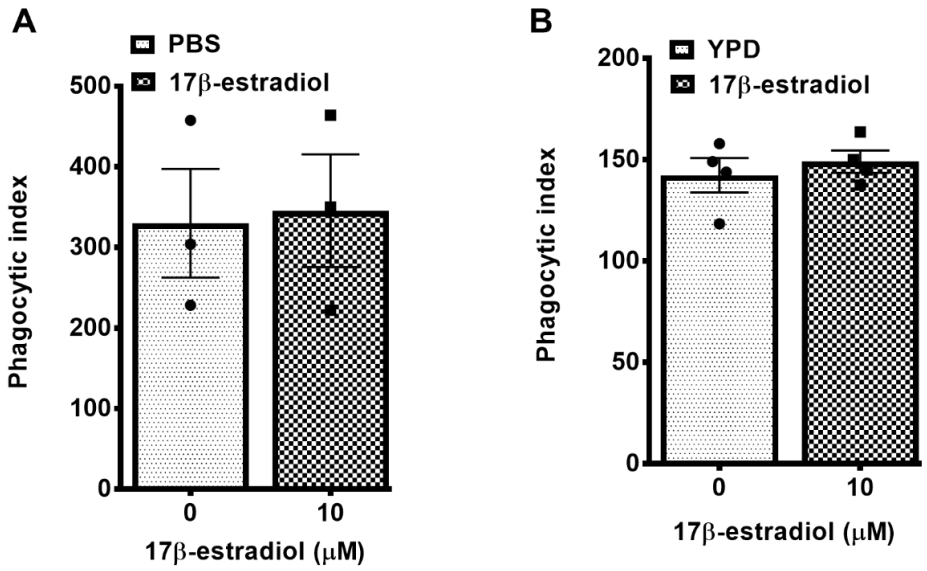

Figure 3.9. Reduced phagocytosis rates are due to adaptation of *C. albicans* to oestrogen and the adaptation is temporary. A) Beads were incubated for 4 h at room temperature in PBS with or without 10 µM 17β-oestradiol. **B**) *C. albicans* (SC5314) was grown in YPD with or without 10 µM oestrogen for up to 4h. Cells were collected, washed, and incubated in fresh YPD for 24 h. Cells were harvested and co-incubated with J774A.1 and phagocytosis quantified. All data represent the mean ± SEM from at least three independent experiments.

Furthermore, a time course phagocytosis assay was conducted where *C. albicans* cells were treated with oestrogen and harvested for phagocytosis at different time points from 5 min to 4 h. There was no significant change observed in phagocytosis of *Candida* cells treated with oestrogen for less than an hour. However, there was a significant reduction in phagocytosis of oestrogen-adapted cells harvested at 1 h and more (Figure 3.10). Once more these results supported the hypothesis that inhibition of phagocytosis could be due to impact of the hormone on the fungus and not the macrophage. In addition, this finding suggested that the fungus undergoes some form of transcriptional response to oestrogen since reduction in phagocytosis is observed after at least 1 h incubation.

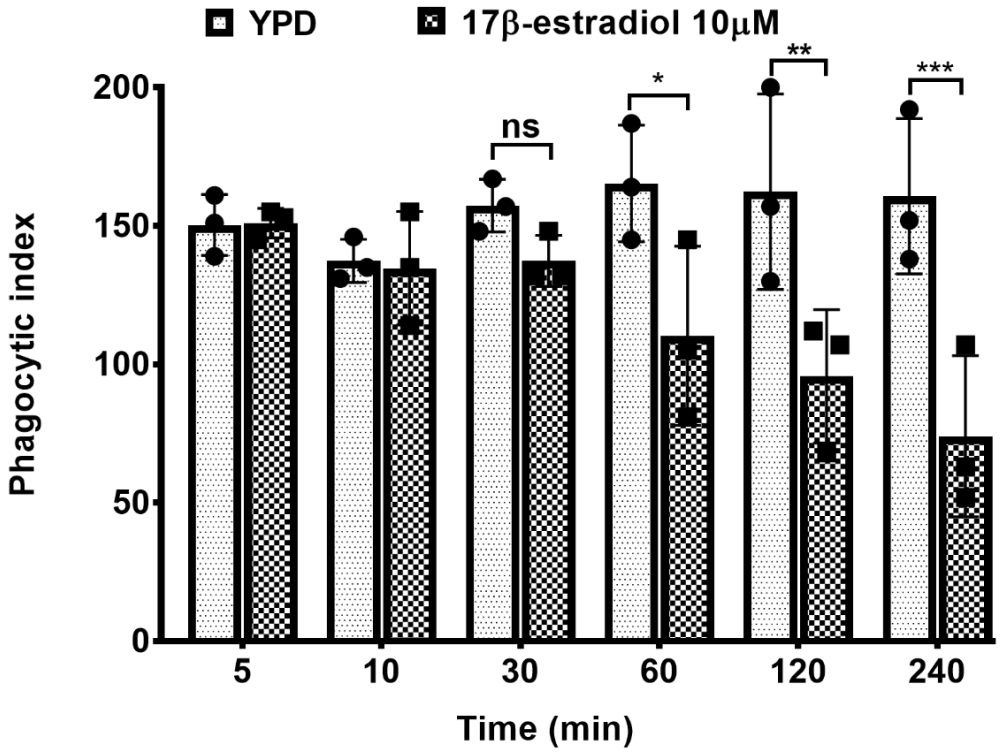

Figure 3.10. Adaptation of *C. albicans* to oestrogen is time dependent. *C. albicans* (SC5314) was grown in YPD with or without 10 μM oestrogen for up to 4h. Cells were harvested at different time points, washed, and incubated with J774A.1 and phagocytosis rates were quantified. Data represent the mean ± SEM from at least three independent experiments. Data were analysed by Two-way ANOVA, and Sidak's multiple comparisons test; *p<0.05, **p<0.01, ***p<0.001.

3.2.6 Adaptation of *C. albicans* to oestrogen is a metabolically active process.

Though the mechanisms by which steroids enter fungal cells are still unclear, research has demonstrated that steroid hormones can rapidly diffuse through membranes (Oren *et al.,* 2004). As such an analysis was performed to establish whether the entry of oestrogen into *C. albicans* requires the cells to be metabolically active or that the adaptation of *C. albicans* to the hormone is also dependent on the metabolic activity of the fungus.

C. albicans cells from overnight culture were resuspended in PBS with or without oestrogen supplementation. Cells were incubated for 4 h on ice and room temperature respectively and phagocytosis was quantified. Unlike those maintained at room temperature (Figure 3.11A), *C. albicans* cells incubated on ice did not exhibit reduction in phagocytosis and the uptake rate was basically the same as its control (i.e., non-oestrogen-adapted cells) (Figure 3.11B).

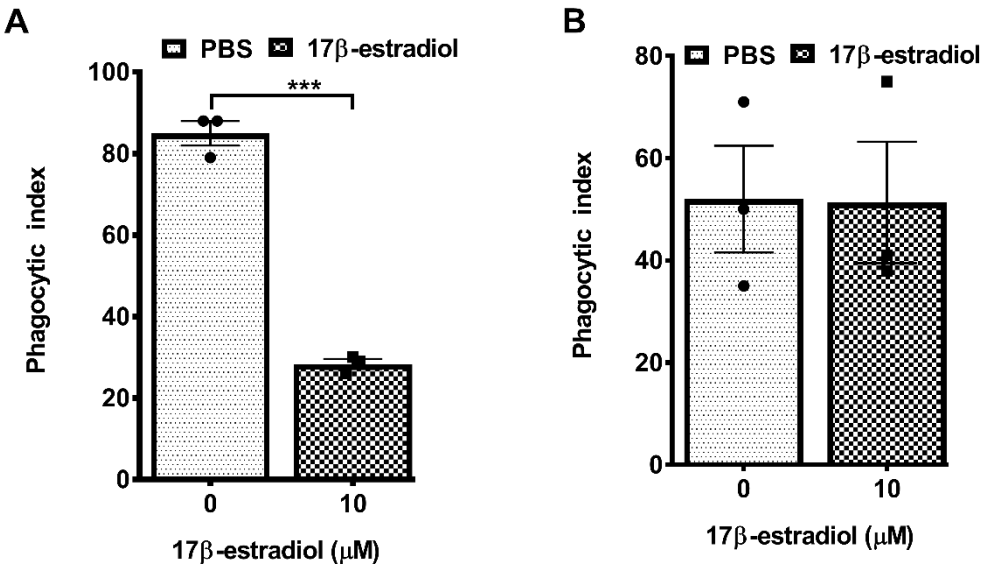

Figure 3.11. Adaptation to oestrogen requires *Candida* cells to be metabolically active. A). *C. albicans* cells from overnight culture were washed and incubated for 4 h in PBS at room temperature with or without 10 μM 17β-estradiol. **B**). *C. albicans* cells from overnight culture were washed and incubated for 4 h in PBS at 4 ^{O}C with or without 10 μM 17β-estradiol. Cells were washed and co-incubated with J774A.1 macrophages and phagocytosis rates quantified. Data represent the mean ± SEM from at least three independent experiments. Data were analysed by unpaired t test; ***p<0.001.

Additionally, we performed a similar experiment but this time using a fluorescently labelled Oestradiol. Contrary to cells grown at room temperature (Figure 3.12A), uptake of oestrogen was not observed in *C. albicans* cells incubated on ice (Figure 3.12B). Therefore, our findings suggest that uptake of oestrogen by *C. albicans* is a metabolically active process, and that the fungus needs to be metabolically active to adapt to the hormone. Nevertheless, this outcome needs to be interpreted with care as this could simply be due to the compromised permeability of the cell as at 4°C the cell membrane becomes rigid.

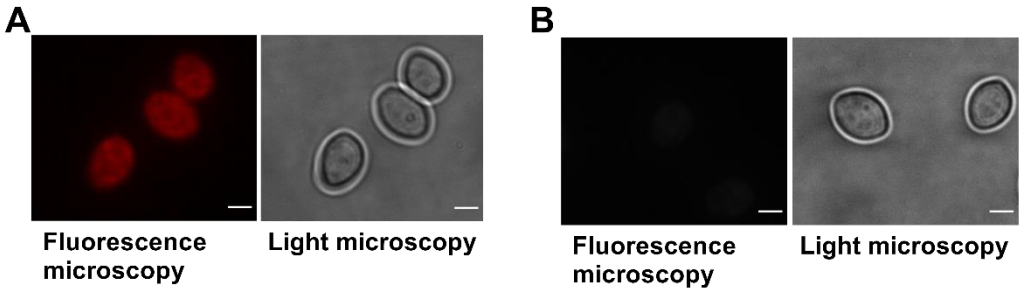

Figure 3.12. An illustration of *C. albicans* oestrogen uptake at room temperature and at 4 °C. A. *C. albicans* cells from overnight culture were washed and incubated for 4 h at room temperature in PBS with or without 10 µM oestradiol glow. **B**. *C. albicans* cells from overnight culture were washed and incubated at 4 °C in PBS with or without 10 µM oestradiol glow. Images were taken by microscopy.

3.3 Discussion

C. albicans is a major fungal pathogen that is metabolically and physiologically flexible to adapt to different environmental conditions *in vitro* and *in vivo* (da Silva Dantas *et al.,* 2016). Correspondingly, the dynamic and complex nature of host niches impact the reproduction (Brown *et al.,* 2014), morphology and host-pathogen interaction of the fungus (da Silva Dantas *et al.,* 2016). Nevertheless, the success of *C. albicans* as a pathogen largely depends on how it circumvents host conditions. This study shows that adaptation of *C. albicans* to oestrogen promotes innate immune evasion. This observation was independent of the influence of the hormone on growth or morphology of the fungus.

3.3.1 Growth of *C. albicans* in oestrogen supplemented YPD broth has no impact on morphology and growth rate of the fungus

Like many other fungal pathogens, the ability of *C. albicans* to colonise and infect a wide range of host environments is dependent on several virulence factors and fitness attributes (da Silva Dantas *et al.,* 2016). Morphogenesis is an important attribute as the yeast-to-hypha switch elicit a morphogenetic response vital in the immunopathogenesis of VVC and stimulation of transition from asymptomatic colonization to symptomatic infection (Peters *et al.,* 2014). Oestrogen is among the several environmental cues that have been implicated in inducing *C. albicans* morphogenesis (Feng *et al.,* 1999, Cheng *et al.,* 2006). Investigations by Feng *et al*. (1999) and Cheng *et al*. (2006) revealed that oestrogen induces filamentation of *C. albicans*.

On the contrary, the current study showed that oestrogen does not significantly alter the morphology of *C. albicans* (Figure 3.1A-C). The differences in findings could owe to a number of factors including the use of YPD media in the current study while the previous studies used charcoal-stripped serum (Cheng *et al.* 2006) and RPMI 1640 medium (Feng *et al.,* 1999). Unlike the current study where YPD broth was used, the use of charcoal-stripped serum or RPMI 1640 medium may have compromised the outcome of these studies since both serum (Feng *et al.,* 1999) and RPMI 1640 medium (Kucharíková *et al.,* 2011) are known to induce yeast-to-hyphae transition in *C. albicans*. YPD was used in the present study to remove any complication of morphogenesis on the phenotype exhibited by oestrogen treatment.

Interestingly, Kurakado *et al*. (2017) reported that oestrogen inhibits yeast-to-hypha transition in *C. albicans*. Here they used media that induces hyphal development and supplement it with 37 µM oestrogen. The fungus was incubated statically for up to 48 h. In contrast to the present study, the concentrations of oestrogen used in our investigation were lower (up to 10 µM) and cells were incubated on agitation for up to 4 h. Also when treated with oestrogen, *C. albicans* increases expression of efflux pumps Cdr1 and Cdr2 (Cheng *et al.,* 2006) to expel the hormone which seem to be toxic. Therefore, the high oestrogen concentration of oestrogen (37 µM) used in this study could be more toxic to the cell and probably affect morphogenesis.

Proliferation of *C. albicans* is required for colonisation and dissemination to cause infection in the female reproductive tract (Gonçalves *et al.,* 2016). Findings of this study demonstrated that oestrogen has no significant impact on the growth rate of *C. albicans in* YPD media. This is in contrast to previous *in vitro* studies that observed that the growth rate of *C. albicans* increased in oestrogen supplemented media (Gujjar *et al.,* 1997, Zhang *et al.,* 2000).

Technical differences in experimental approach may contribute to the variation in results. In the current study, absorbance readings generated from a plate reader were used to determine the growth rates of *C. albicans* whereas the previous study examined the impact of the hormone on growth rate by determining viable *Candida* colony counts (Zhang *et al.,* 2000). Furthermore, the concentrations of oestrogen used in the current and previous study (Gujjar *et al.,* 1997) are different. Steroid hormones can exhibit different effects on fungus depending on their concentration. At a lower concentration, progesterone promoted growth of *A. fumigatus*, while at a higher concentration the hormone inhibited growth (El-Sherif *et al.,* 1975). In addition, a study that used a similar approach to the current investigation also showed that oestrogen does not have an influence on growth of *Paracoccidioides brasiliensis* (Restrepo *et al.,* 1984). Therefore, it is not surprising to note that findings from this study are different from previous investigations.

3.3.2 Oestrogen-adapted *C. albicans* cells evades innate immunity.

VVC is an inflammatory disease affecting the majority of healthy women in their reproductive age (Sobel 2014). Oestrogen has been implicated in pathogenesis of *Candida* vaginitis (Sobel 2014). However, there is little understanding on how exposure of the fungus to the hormone in the female reproductive tract could impact its ability to cause the disease. Our study revealed that adaptation of *C. albicans* to oestrogen *in vitro* attenuated macrophage and neutrophil phagocytosis. Interestingly, this observation was exhibited by physiological type of oestrogen as well as the synthetic form used in oral contraceptive pills. This discovery may improve our basic knowledge on understanding the pathogenesis of VVC.

While *C. albicans* is exposed to the fluctuating concentration of oestrogen in the vagina mucosa, VVC is common during pregnancy and the luteal phase of the menstrual cycle when oestrogen levels are high (Sobel 2014). In addition, VVC infections are prevalent in women taking oestrogen oral contraceptive pills and those on hormone replacement therapy (Gonçalves *et al.,* 2016). Among other factors, data from this investigation suggest that the elevated levels of oestrogen during these periods promote adaptation of *C. albicans* to the hormone thereby enhancing its ability to escape uptake by phagocytes. Considering that phagocytosis is essential in clearing fungal pathogens this phenomenon could promote survival and persistence of *C. albicans* to proliferate and cause symptomatic infection.

Excessive recruitment of neutrophil in the vagina mucosa is one of the clinical features of VVC however this does not lead to successful clearance of the fungus (Fidel *et al.,* 2004). Therefore, apart from neutrophil anergy (Yano *et al.,* 2018), the present discovery may shed more light as to why *C. albicans* is not efficiently cleared during VVC.

3.3.3 Not all *Candida* species evade innate immunity after adaptation to oestrogen.

C. albicans is the predominant cause of VVC followed by *C. glabrata* and rarely *Candida tropicalis, C. parapsilosis* and *C. krusei* (Sobel 2007). Study on other yeasts cells showed that only *C. tropicalis* and *S*. *cerevisiae* evaded innate immunity after adaptation to oestrogen. This is not a strange phenomenon as previous reports have shown that fungus adapt differently to various environmental conditions. For instance, in response to low pH, *C. albicans* and *C. tropicalis* unmasks β-glucan while *C. glabrata* and *S. cerevisiae* does not (Sherrington *et al.,* 2017). Nevertheless, the present finding is slightly surprising since *C. glabrata* and *S. cerevisiae* are quite closely related while *C. albicans* and *S. cerevisiae* are not. Thus, further work to determine the mechanism behind variation to oestrogen response among *Candida* species is required.

3.4 Conclusion

To conclude, this chapter has demonstrated that apart from directly rendering the female reproductive tract susceptible to *Candida* infections, oestrogen may directly impact the fungus to become more virulent and possibly promote transition to symptomatic infection.

4 The effect of *C. albicans* adaptation to oestrogen on cell wall remodelling and immune evasion.

4.1 Background

Fungal cell walls are unique and dynamic structures that are necessary for cell viability, morphogenesis, and pathogenesis. The fungal cell wall is a highly complex organelle whose structure and composition significantly impacts the ecology of the fungus (Gow *et al.,* 2017). The fungal cell wall is critically regulated in response to environmental niches and imposed stresses (Gow *et al.,* 2017).

C. albicans cell wall is largely composed of polysaccharides whereby mannan and mannoproteins are in the outer layer covering the inner components such as β-glucan, a major fungal PAMP. The shielding of β-glucan by mannan and mannoproteins is an immune evasion strategy preventing recognition by Dectin-1. Several studies have demonstrated the importance of this strategy in protecting the fungus. For example, *C. albicans* mutant strains lacking *O*- and *N*-linked mannan are more readily phagocytosed and killed (McKenzie *et al.,* 2010). Similarly, adaptation of *C. albicans* to low pH promotes unmasking of β-glucan and this promotes phagocytosis of the fungus and induction of pro-inflammatory response (Sherrington *et al.,* 2017). Furthermore, *C. albicans* hyphae induces a weaker pro-inflammatory innate immune response due to limited exposure of β-glucan (Gantner *et al.,* 2005, van der Graaf *et al.,* 2005).

Additionally, formation of neutrophil extracellular traps (NETs) against *C. albicans* induces cell wall remodelling in the fungus leading to exposure of β-glucan and subsequent increase in fungus immune recognition (Hopke *et al.,* 2016). Therefore, host niches are vital in inducing cell wall remodelling in *C. albicans* and influence how the fungus interacts with the host.

Fungal cell wall proteins can affect the composition and structure of fungal cell wall and subsequently influence innate immune recognition and virulence of the pathogen. For instance, loss of the *RIM101* transcription factor leads to reduced virulence in *C. albicans* partly due to decrease in expression of cell wall proteins (Nobile *et al.,* 2008). In addition, increased expression of Pga22 can promote development of *C. albicans* biofilms (Cabral *et al.,* 2014). Thus, increased, or decreased expression levels of fungal cell wall proteins can influence the host pathogen interaction. The aim of this chapter is to investigate the role of oestrogen in fungal cell wall remodelling and its subsequent impact on immune evasion.

4.2 Results

4.2.1 Oestrogen-induced immune evasion is not associated with changes in cell wall carbohydrates.

Fungal cell wall carbohydrates are important in influencing the host-pathogen interaction (Arana *et al.,* 2009). However, some host niches are known to promote immune evasion by inhibiting recognition of cell wall carbohydrate PAMPs.

For instance, adaptation of C*. albicans* to lactate and hypoxia triggers β-glucan masking thereby promoting immune evasion (Ballou *et al.,* 2016, Pradhan *et al.,* 2018). To ascertain whether *C. albicans* adaptation to oestrogen affects phagocytosis through modulation of cell wall carbohydrates, levels of mannan, β-glucan and chitin were established post hormone treatment. Quantification of total mannan, β-glucan and chitin were determined by FACs following staining with Con A, Aniline blue and Calcofluor White (CFW) respectively. Levels of total mannan, β-glucan and chitin remained unchanged regardless of oestrogen treatment (Figure 4.1).

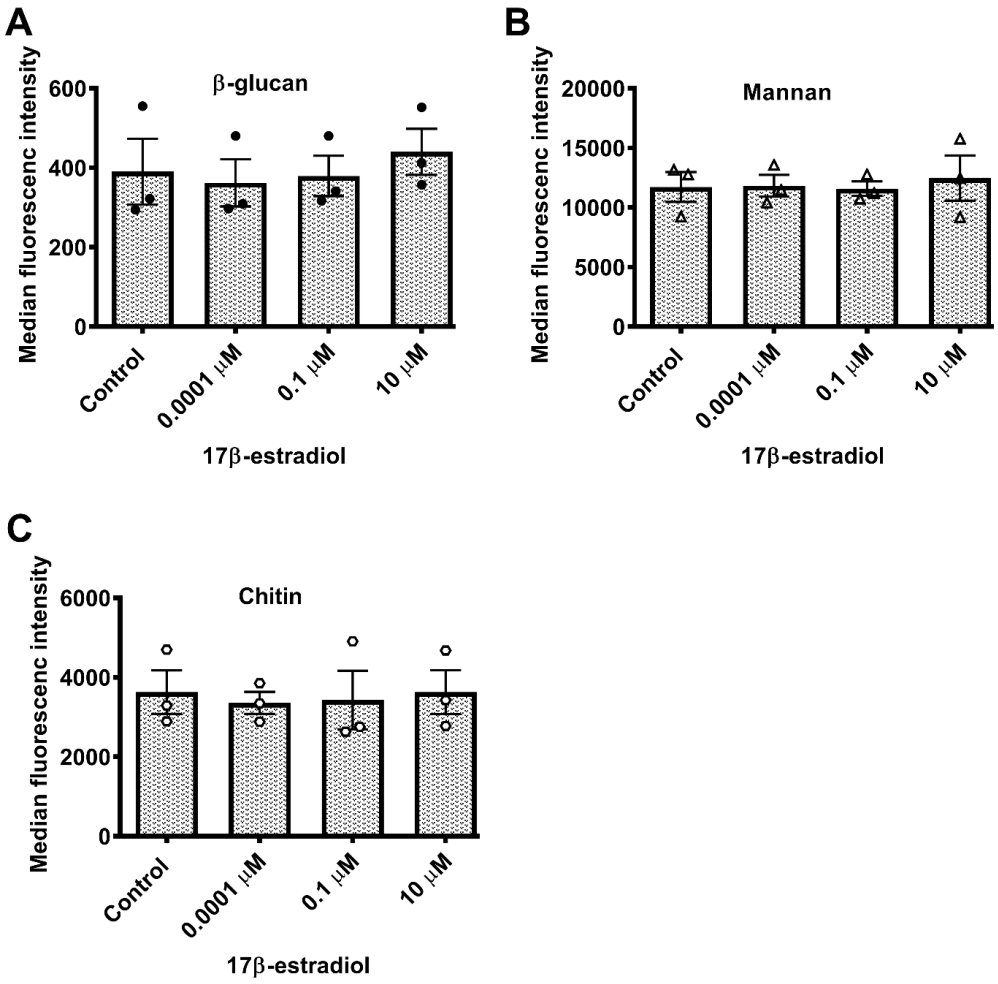

Figure 4.1. Reduced phagocytosis rates are not due to gross changes in cell wall carbohydrates. *C. albicans* cells were grown for 4 h in YPD with or without 17β-oestradiol complementation. Cells were harvested, washed in PBS, fixed with 4% PFA and stained for (**A**) total mannan (**B**) total glucan and (**C**) total chitin. Staining was quantified by flow cytometry and median fluorescence intensities (MFI) determined. All data represent the mean ± SEM from at least three independent experiments.

4.2.2 The oestrogen-dependent innate immune escape is not due to inhibition of recognition by Dectin-1.

Dectin-1 is a pattern-recognition receptor that detects β-1,3-glucan on *C. albicans* and is therefore important in providing immune defence against the fungus (Brown *et al.,* 2001). Dectin-1 is expressed on immune cells including neutrophils, monocytes, dendritic cells and macrophages (Monie 2017). *C. albicans* adaptation to some host environmental cues has been reported to promote reduction in phagocytosis through inhibition of Dectin-1 recognition by masking of exposed β-glucan (Ballou *et al.,* 2016, Pradhan *et al.,* 2018). To establish if reduction of phagocytosis was dependent on Dectin-1, the association of oestrogen-adapted *C. albicans* cells to Dectin-1-expressing fibroblast cells was quantified. Dectin-1 bound to oestrogen adapted *C. albicans* cells at rates similar to YPD control cells, thus signifying that the observed changes in phagocytosis rates are not a consequence of reduced recognition by Dectin-1 (Figure 4.2). To complement this finding, the quantity of surface exposed β-glucan or chitin following adaptation to oestrogen was assessed by FC Dectin-1 antibody detection staining and wheat germ agglutinin (WGA) staining, respectively. FACs analysis confirmed that adaptation of *C. albicans* to oestrogen had no impact on surface exposure of either β-glucan or chitin (Figure 4.3).

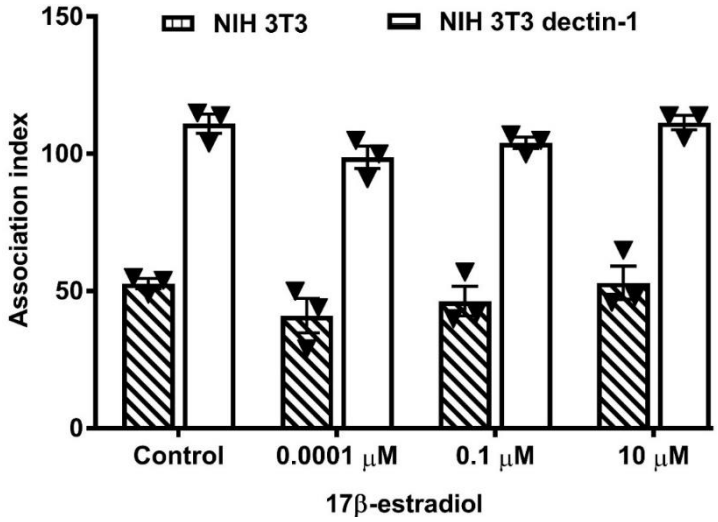

Figure 4.2. Reduction in phagocytosis is not dependent on Dectin-1 recognition. *C. albicans* cells were grown for 4 h in YPD with or without 17β-oestradiol. Cells were harvested, washed in PBS, and incubated with NIH 3T3 fibroblasts cells expressing Ddectin-1. The association index of *C. albicans* cells was quantified using ImageJ. All data represent the mean ± SEM from at least three independent experiments

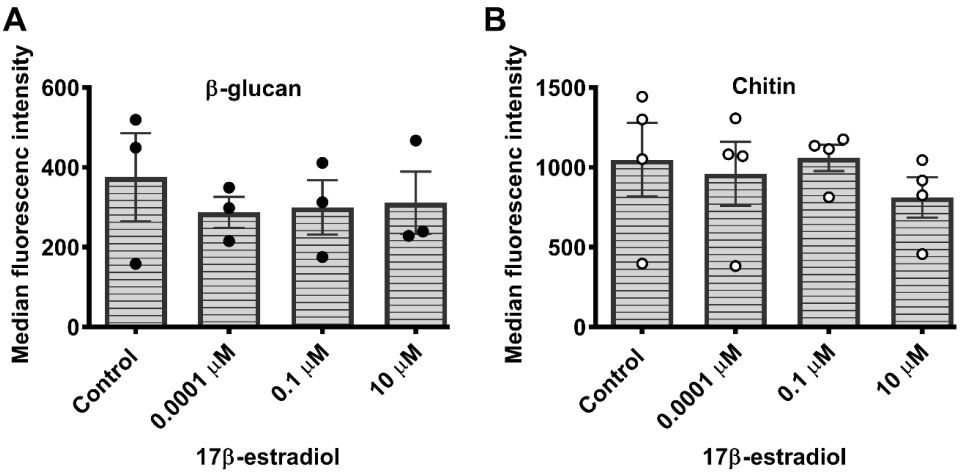

Figure 4.3. Adaptation to oestrogen does not induce exposure of cell wall carbohydrates. *C. albicans* cells were grown for 4 h in YPD with or without 17β-oestradiol. Cells were harvested, washed in PBS, fixed with 4% PFA and stained for (**A**) exposed β-glucan and (**B**) exposed chitin levels. Staining was quantified by flow cytometry and MFI determined. All data represent the mean ± SEM from at least three independent experiments.

4.2.3 Inhibition of phagocytosis in oestrogen-adapted *C. albicans* cells is not linked to changes in biosynthesis of ergosterol.

Structurally, after the fungal cell wall is the plasm membrane which contains ergosterol, the most dominant sterol in fungal cell membranes where its main function is to regulate permeability and fluidity (Douglas *et al.,* 2014). Ergosterol is the target of most clinically relevant antifungals because it has critical functions, distinctive structural properties, and peculiar biosynthetic steps (Dhingra *et al.,* 2017). Furthermore, *In vitro* studies have demonstrated that ergosterol inhibits phagocytosis in lipopolysaccharide (LPS)-induced RAW264.7 macrophages (Yuan *et al.,* 2019). Therefore, the involvement of fungal ergosterol in the oestrogen induced reduction of phagocytosis was investigated by analysing the level of ergosterol in oestrogen-adapted *C. albicans* cells. Quantification of ergosterol remained unchanged irrespective of adaptation to oestrogen suggesting that it is not associated with attenuation of phagocytosis (Figure 4.4).

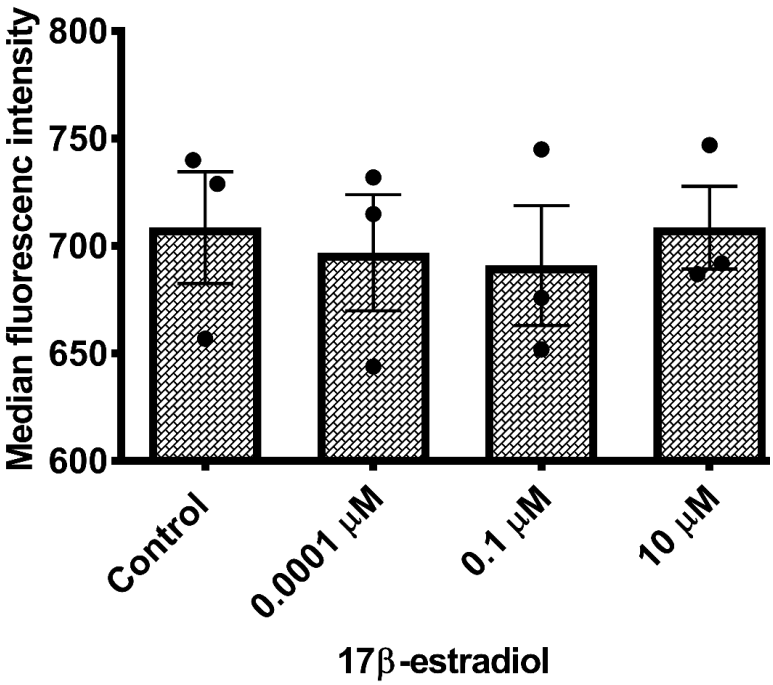

Figure 4.4. Adaptation to oestrogen does not influence the synthesis of Ergosterol. *C. albicans* cells were grown in YPD with or without 17β-oestradiol for 4 h. Cells were harvested, washed in PBS, fixed with 4% PFA and stained with filipin stain for Ergosterol using filipin levels. Staining was quantified by flow cytometry and MFI determined. All data represent the mean ± SEM from at least three independent experiments.

4.2.4 Adaptation of *C. albicans* to oestrogen triggers a weak transcriptional response.

RNA sequencing (RNA-Seq) is an essential bioscience technique that provides understanding of the transcriptome of a cell. Among other things, RNA-seq can allow quantification of expression levels of genes in a cell. Therefore, important genes expressed during different cellular activities or environments can be detected using RNA-seq technique. Thus, an investigation was performed to examine the effect of oestrogen on *C. albicans* transcriptome. RNA sequencing was performed on *C. albicans* cells adapted to oestrogen for 4 h to identify differentially regulated genes.

Irrespective of the strong effect on the host-pathogen interaction, oestrogen only displayed a mild impact on the transcriptome of *C. albicans* with the majority of regulated genes shown to be involved in metabolic and anabolic processes (Table S4.1). However, since the process of phagocytosis is mostly initiated by interaction between pattern recognition receptors (PRRs) on phagocytes and pathogen-associated molecular patterns (PAMPs) on fungal cell surface, analysis of RNA-seq data was concentrated on genes known to code for cell surface proteins or those involved in cell wall biogenesis. After this analysis, a few genes were identified as mildly differentially regulated (Table 4.1).

Table 4.1. Gene expression analysis for cell surface proteins in *C. albicans* cells adapted to oestrogen

Gene name	Fold change	Adjusted p-value
EBP1	1.86	7.85E-09
CDR1	1.65	0.0000396
SOD6	1.56	0.000749
ALS2	1.31	0.01
UTR2	1.27	0.02
FGR23	1.26	0.03
GPD2	1.21	0.06
ECM331	-1.21	0.03
HSP21	-1.22	0.07
PGA4	-1.23	0.03
CSA1	-1.25	0.03
SAP10	-1.27	0.03
PLB4.5	-1.27	0.02
PGA23	-1.31	0.02
PGA10	-1.32	0.01
RBT4	-1.32	0.01
HSP104	-1.35	0.00253
PHR1	-1.36	0.03
IFF4	-1.37	0.02
CSP37	-1.37	0.00229
GPH1	-1.43	0.000767
XOG1	-1.46	0.000325
HSP70	-1.47	0.000249
CSH1	-1.55	0.0000623
MET6	-1.62	3.51E-08
PGA26	-1.65	0.07
PHO100	-1.77	0.05
DDR48	-1.77	0.000000264
IFF9	-1.89	0.02
IHD1	-2.4	5.55E-16
PGA15	-2.43	0.06
PGA46	-2.44	0.06
HWP1	-3.72	0.000928

4.2.5 *CDR1* gene does not facilitate the oestrogen driven innate immune escape in *C. albicans*.

Previous studies have shown that *C. albicans CDR1* and *CDR2* genes are known to transcriptionally respond to oestrogen. Treatment of *C. albicans* with oestrogen leads to increased expression of *CDR1* and *CDR2* (Krishnamurthy *et al.,* 1998, Zhang *et al.,* 2000, Micheli *et al.,* 2002, Cheng *et al.,* 2006). *CDR1* and *CDR2* codes for the efflux pumps Cdr1p and Cdr2p respectively (Sanglard *et al.,* 1997, Krishnamurthy *et al.,* 1998). β-oestradiol stimulates transcription of *TAC1* which subsequently triggers the transcription of *CDR1* and *CDR2* (Coste *et al.,* 2004). The Cdr1p and Cdr2 confer antifungal resistance by pumping the drugs out of the cell. Therefore, the increased transcription of *CDR1* and *CDR2* following adaptation to oestrogen most probably heightens the production of efflux pumps, which clears the hormone from *C. albicans* cell (Krishnamurthy *et al.,* 1998).

Thus, among the transcriptionally upregulated genes following adaptation to oestrogen, *CDR1* was the first to be examined for its role in influencing innate immune evasion. Deletion of *CDR1* alone or both *CDR1* and *CDR2* had no impact on *C. albicans* host-pathogen interaction (Figure 4.5). Therefore, this suggests that oestrogen activates other genes/proteins in *C. albicans* to mediate the immune evasion. In addition, loss of oestrogen through increased expression of Cdr1 and Cdr2 efflux pumps may not be sufficient to clear the hormone and inhibit the immune circumvention.

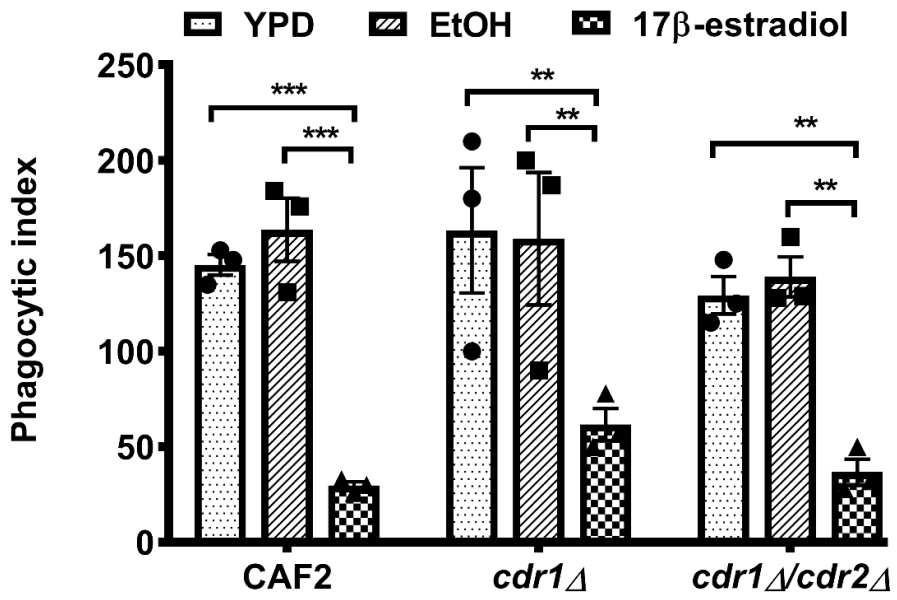

Figure 4.5*. CDR1* and *CDR2* genes do not mediate the oestrogen dependent reduction of *C. albicans*. *C. albicans* cells were grown for 4 h in YPD with or without 10 µM 17β-oestradiol. *Cdr1*Δ and *cdr1*Δ*/cdr2*Δ *C. albicans cells* were harvested, washed in PBS and co-incubated with J774A.1 macrophages for 45 min. Phagocytosis rates were quantified. All data represent the mean ± SEM from at least three independent experiments. Data were analysed by Two-way ANOVA and Tukey's multiple comparisons test; **p<0.01, ***p<0.001.

Furthermore, it has been shown that adaptation of *C. albicans* to oestrogen upregulates *TAC1* and *STP4* genes. These genes encode Tac1 and Stp4 which are transcription factors for *CDR1* and *CDR2* respectively (Coste *et al.,* 2004, Cheng *et al.,* 2006). Therefore, *tac1*Δ and *stp4*Δ transcription factors were assessed for their role in reduction of *C. albicans* phagocytosis. Following treatment with oestrogen, *tac1*Δ and *stp4*Δ strains were co-incubated with macrophages and phagocytosis rates quantified. Tac1 was found not to be involved in mediating the oestrogen-induced innate immune escape as adaptation of *tac1*Δ to oestrogen also resulted into inhibition of phagocytosis just like the parental control strain (Figure 4.6).

However, deletion of *STP4* led to loss of phenotype as adaptation to oestrogen did not result into reduction of phagocytosis in *stp4Δ*. This suggested that *STP4* is essential in mediating innate immune evasion (Figure 4.6). Further analysis of the *stp4Δ* mutant revealed that this strain exposed significantly more β-glucan than the wild type strain (Figure 4.7) as such the yeast cells were more readily phagocytosed than the parental strain. Therefore, it was hypothesized that the loss of the oestrogen induced phenotype in the *stp4Δ* strain was attributed to the high levels of β-glucan that overshadowed the impact of oestrogen on *C. albicans*.

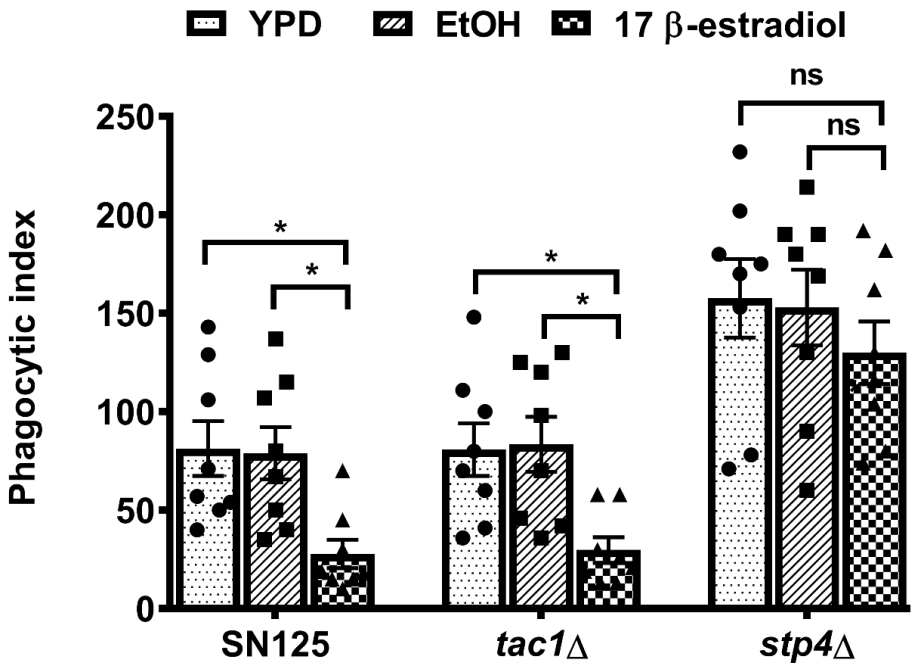

Figure 4.6. Tac1 and Stp4 transcriptions factors are not critical in facilitating the oestrogen dependent reduction of *C. albicans*. Tac1Δ and stp4Δ *C. albicans* cells were grown for 4 h in YPD with or without 10 µM 17β-oestradiol. *Candida* cells were harvested, washed in PBS and co-incubated with J774A.1 macrophages for 45 min. Phagocytosis rates were quantified using ImageJ. All data represent the mean ± SEM from at least three independent experiments. Data were analysed by Two-way ANOVA and Dunnett's multiple comparisons test; *p<0.05.

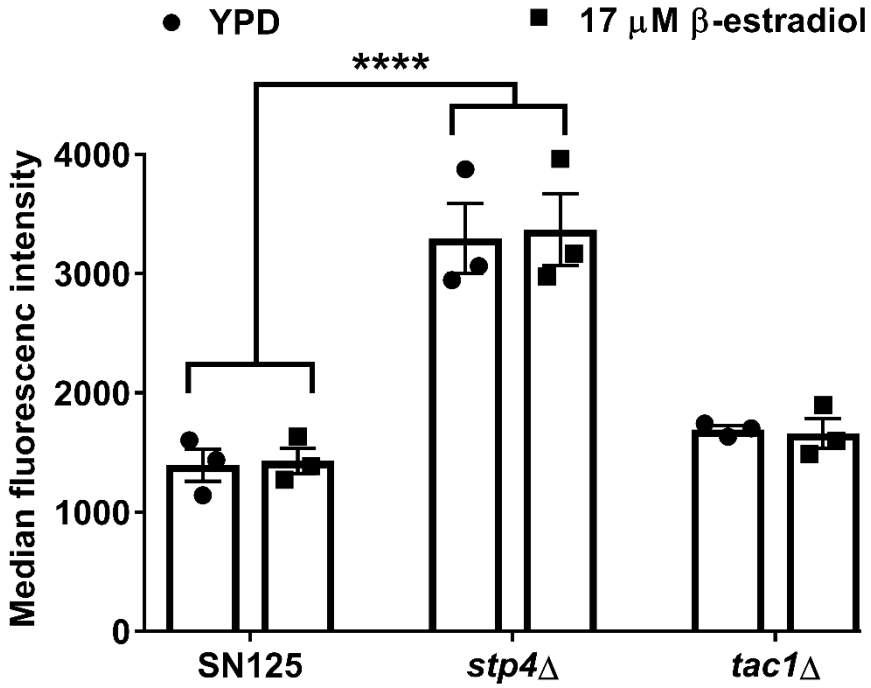

Figure 4.7. Deletion of *STP4* promotes exposure of β-glucan. *C. albicans* cells were grown in YPD with or without 10 μM 17β-oestradiol for 4 h. Cells were harvested, washed in PBS, fixed with 4% PFA and stained for exposed β-glucan levels. Staining was quantified by flow cytometry and MFI determined. All data represent the mean ± SEM from at least three independent experiments. Data were analysed by Two-way ANOVA and Sidak's multiple comparisons test; ****p<0.0001.

4.2.6 Gpd2 mediates the *C. albicans* oestrogen dependent innate immune evasion.

Considering that *Candida* Gpd2 was previously identified as an immune evasion protein (Luo *et al.,* 2013), *GPD2* gene was evaluated on its role in influencing the oestrogen-dependent immune evasion. *GPD2* was deleted in *C. albicans,* and phagocytosis rates determined after treatment with oestrogen. Deletion of *GPD2* prevented oestrogen dependent inhibition of macrophage phagocytosis (Figure 4.8A), confirming that *GPD2* is required for oestrogen dependent innate immune evasion.

To further ascertain the role of Gpd2 in facilitating the oestrogen driven innate immune evasion, *GPD2* overexpressed *C. albicans* strain was co-incubated with macrophages. Over-expression of *GDP2* resulted in reduced phagocytosis rates irrespective of oestrogen treatment (Figure 4.8B), thereby confirming that enhanced expression of *GDP2* is sufficient to promote *C. albicans* innate immune evasion.

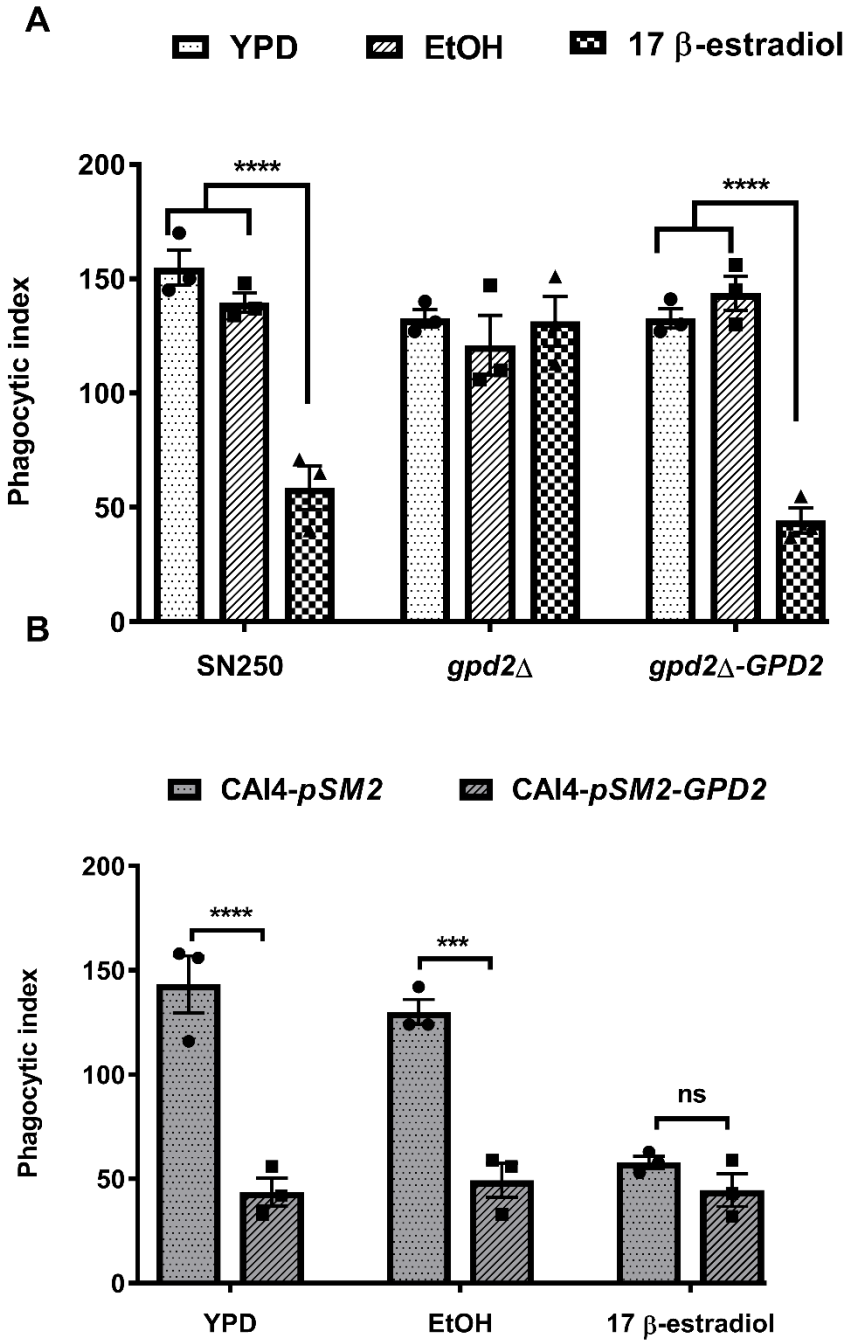

Figure 4.8. **Oestrogen-driven immune evasion is dependent on Gpd2.** *C. albicans* strains (**A**) SN250, *gpd2*Δ, *gpd2*Δ-*GPD2* (Gpd2 reconstituted strain) and (**B**) CAI4-pSM2 and CAI4-pSM2-*GPD2* were grown for 4 h in YPD with or without 10 μM 17β-oestradiol, harvested and co-incubated with J774.1A cells. Phagocytosis rates were quantified using ImageJ. All data represent the mean ± SEM from at least three independent experiments. Data were analysed by Two-way ANOVA and Tukey's multiple comparisons test; ***p<0.001, ****p<0.0001.

4.2.7 Some level of *GPD2* expression is required to initiate the oestrogen-driven innate immune evasion.

After establishing the role of *GPD2* in facilitating the oestrogen-dependent inhibition of *C. albicans*, a qPCR experiment was performed on oestrogen adapted cells to validate the effect of oestrogen on Gpd2 expression. Findings demonstrated that adaptation to oestrogen results into a nearly two-fold increase in *GPD2* expression (Figure 4.9).

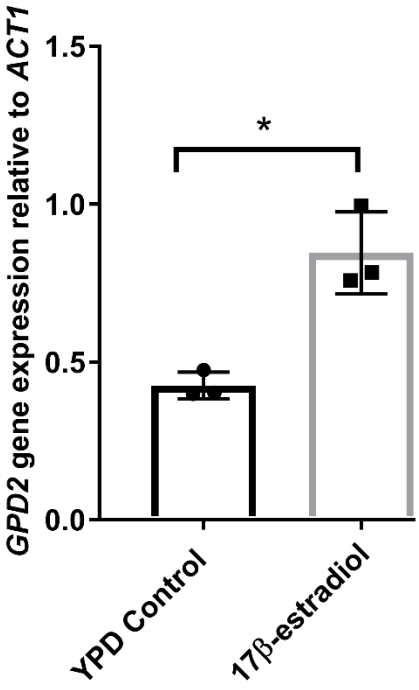

Figure 4.9. Adaptation to oestrogen promotes upregulation of *GPD2*. *C. albicans* cells from overnight culture were grown for 4 h in YPD with and without 10 μM 17β-oestradiol. Cells were harvested for total RNA extraction. Expression of *GPD2* was quantified by RT-PCR (n=3). *GPD2* expression levels are shown relative to *ACT1*. Data were analysed by Unpaired t-test; *p<0.05.

Furthermore, analysis was performed to detect transcription factors that facilitate the expression of *GPD2*. The promoter region of *GPD2* was analysed for potential transcription factors using the pathoyeastract program. Consequently, the program identified 17 transcription factors linked with the promoter region of *GPD2*. Among the detected transcription factors, 10 (i.e., Sfl1, Rpn4, Tye7, Gal4, Ndt80, Csr1, Rob1, Ssn6 and Bcr1) have been deleted in *C. albicans* and are available in the Nobel transcription factor knockout library. Thus, the mutants for the 10 transcription factors were studied for their role in regulating *GPD2*. To study the role of these transcription factors in the regulation of *GPD2*, qPCR was performed to quantify the expression levels of *GPD2* in the mutants. Findings revealed that loss of *BCR1* and *ROB1* had a significant effect on expression of *GPD2*, causing a nearly threefold reduction in *GPD2* expression in the mutants in comparison to the wild type control (Figure 4.10).

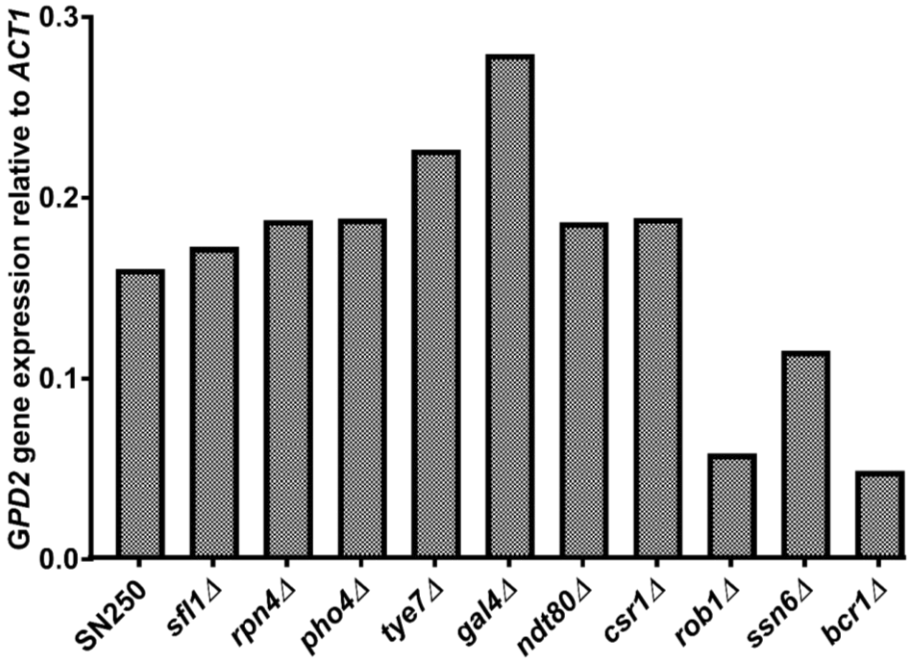

Figure 4.10. Transcription factors involved in the expression of *GPD2*. *C. albicans* cells (SN250, sfl1Δ, rpn4Δ, pho4, tye7Δ, gal4Δ, ndt80Δ, csr1Δ, rob1Δ, ssn6Δ and bcr1Δ) from overnight culture were grown for 4 h in YPD with 10 μM 17β-oestradiol and harvested for total RNA extraction. Expression of *GPD2* was quantified by RT-PCR (n=1). *GPD2* expression levels are shown relative to *ACT1*.

Next, an investigation was carried out to determine whether the reduction in expression levels of *GPD2* in the *bcr1*Δ and *rob1*Δ mutants could negatively influence the oestrogen response in *C. albicans*. Thus, the *bcr1*Δ and *rob1*Δ mutants were treated with oestrogen and co-incubated with macrophages. Unlike *rob1*Δ mutant, phagocytosis rates of *bcr1*Δ mutant were restored as in the wild-type strain (Figure 4.11). This outcome suggested that Bcr1 is the key regulator for transcription of *GPD2* and that some level of *GPD2* expression is essential for the oestrogen-dependent immune evasion to occur.

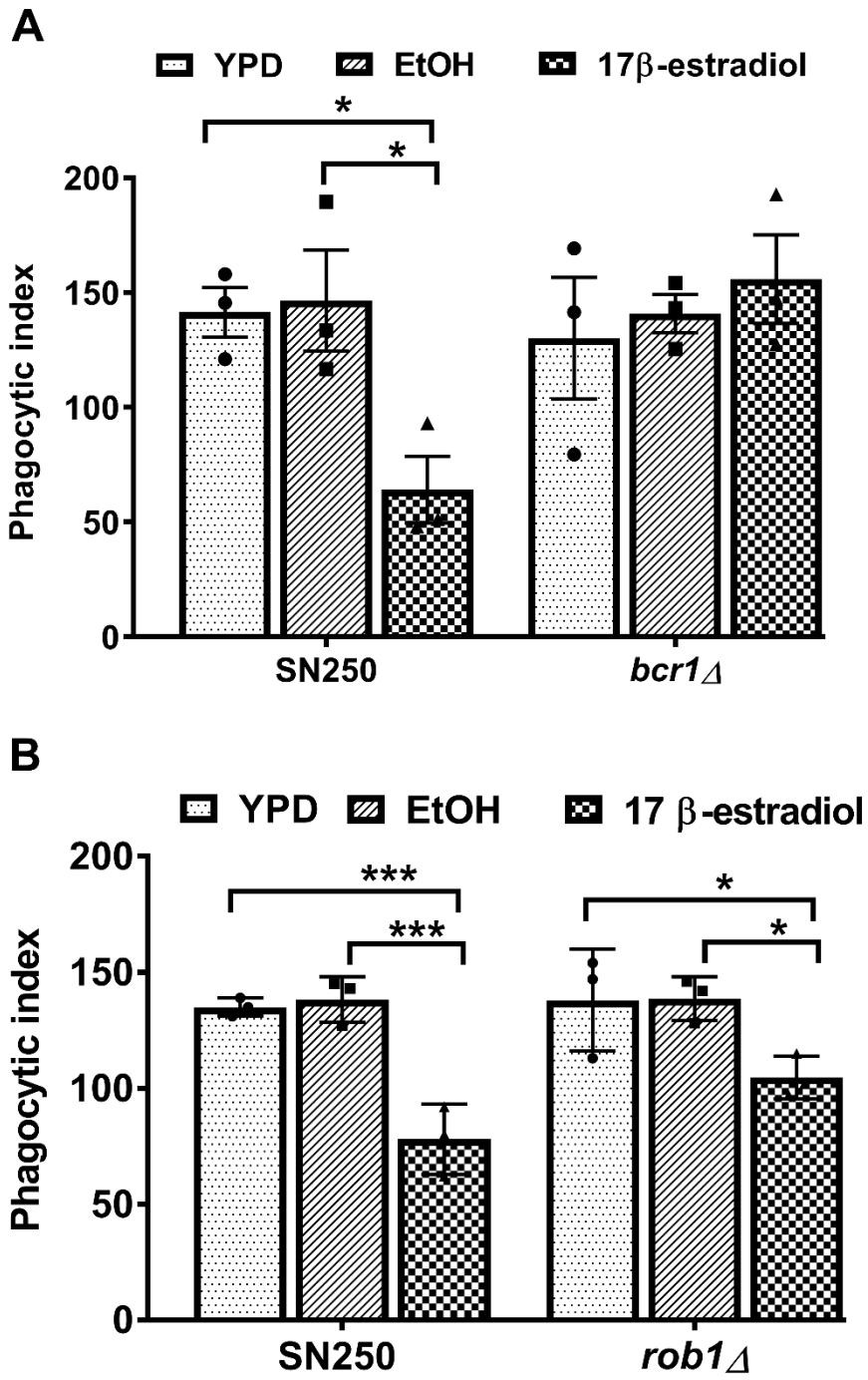

Figure 4.11. Increased expression of *GPD2*, regulated by Bcr1 is required for innate immune evasion to occur. *C. albicans* cells (A) SN250, bcr1Δ and (B) SN250, rob1Δ were grown for 4 h in YPD with or without 10 μM 17β-oestradiol. Yeast cells were harvested, washed in PBS, co-incubated with J774.1A macrophages for 45 min and phagocytosis rates quantified. All data represent the mean ± SEM from at least three independent experiments. Data were analysed by Two-way ANOVA and Tukey's multiple comparisons test; *p<0.05, ***P<0.001.

4.3 Discussion

4.3.1 *C. albicans* cell wall polysaccharides are not essential in mediating the oestrogen-dependent immune evasion.

The fungal cell wall plays a critical role in interaction with the host *in vivo*. Among other functions, the components of the fungal cell wall provide protection by manipulating host immune response to promote fungal growth and dissemination within the host (Poulain *et al.,* 2004, Galán-Díez *et al.,* 2010, Ifrim *et al.,* 2016). β-glucan is one of the key fungal PAMPs that elicit a strong immune response to protect the host (Qin *et al.,* 2016). As such, *Candida* species ensure to mask β-glucan as any disturbance on biosynthesis or structural organisation exposes the carbohydrate and increases the ability of the host immunity to recognise and attack the fungus (Granger 2018). For instance, cell wall damage by antifungals or host enzymes promote phagocytosis by macrophages through enhancing beta-glucan exposure (Wheeler *et al.,* 2008). Also adaptation of *C. albicans* to low pH modifies the ultrastructure of the fungus leading to exposure of β-glucan and enhance uptake by phagocytes (Sherrington *et al.,* 2017). Thus, β-glucan masking is particularly important in promoting immune evasion.

Mannoproteins form the most outer layer of the fungal cell wall. They are important in initiating fungal interaction with the host to stimulate and modulate immune response against *Candida* (Brown *et al.,* 2012, Shibata *et al.,* 2012, Paulovičová *et al.,* 2015). Mannoproteins mask the β-glucan layer thereby preventing immune recognition and this greatly impacts the capacity of phagocytes to phagocytose and kill *Candida* cells (Galán-Díez *et al.,* 2010, Bain *et al.,* 2014). β-glucan masking also enhances resistance to the classical and alternative complement systems resulting in unsuccessful activation of the immune system (Zhang *et al.,* 1998, Boxx *et al.,* 2009, Boxx *et al.,* 2010).

Chitin is another cell wall polysaccharide that plays a vital role in host-fungal interaction. Chitin inhibit recognition of *C. albicans* by PBMCs and murine macrophages thus resulting in a substantial reduction in cytokine production (Mora-Montes *et al.,* 2011). Therefore, host environment that promotes masking or downregulation of the synthesis of *C. albicans* cell wall polysaccharides is critical in promoting fungal innate immune evasion. Consequently, the observation that adaptation of *C. albicans* to oestrogen did not influence the biosynthesis or organisation of mannoproteins, β-glucan or chitin confirms that the carbohydrates had no role in mediating the oestrogen-induced reduction in phagocytosis.

4.3.2 The oestrogen-driven innate immune escape is mediated by Gpd2.

Similar to a previous study (Cheng *et al.,* 2006), global transcriptional analysis of oestrogen adapted *C. albicans* cells revealed that only few genes were differentially regulated by the hormone. Cheng *et al*., 2006 revealed that most of the genes differentially regulated were associated with fungal morphogenesis. However, the present investigation discovered that most differentially regulated genes were linked to metabolism and redox reactions (Table 4.1). The difference in outcomes could be attributed to growth settings since the current study used YPD which does not promote hyphal formation, whereas Cheng *et al*., 2006 utilised RPMI which stimulates generation of hyphae thereby inducing expression of a different set of genes (Kucharíková *et al.,* 2011).

Analysis of cell surface genes identified Gpd2 as one of the mildly differentially regulated genes (Table 4.2). As a previously known immune evasion protein, further analysis was conducted to understand its role in the oestrogen driven innate immune evasion. Gpd2 was identified as essential in facilitating the oestrogen dependent innate immune escape. It was also observed that a threshold level of *GPD2* expression is required for the oestrogen driven innate immune evasion to take effect. Nevertheless, qPCR analysis showed that the increase in *GPD2* expression in response to oestrogen was moderate. Therefore, this suggests that the mechanism for the oestrogen mediated innate immune evasion may as well involve other strategies.

In addition, since Gpd2 has solely been recognized as a cell wall protein under a particular environment, the protein might be localised to the cell wall in response to oestrogen and initiate innate immune evasion.

4.4 Conclusion

The oestrogen dependent innate immune evasion strategy is mediated by Gpd2 however it could be a multifactorial process considering that there were also other cell surface genes differentially regulated. The discovery that Gpd2 facilitates immune evasion in oestrogen adapted *C. albicans* cells is vital as it opens opportunities for further understanding of the virulence mechanisms of *C. albicans*. Besides, this might likewise allow the generation of novel strategies to counter *C. albicans* and combat *C. albicans* infections.

5 The role of complement in *C. albicans* oestrogen response

5.1 Background

The process of phagocytic response to microbes involves the stimulation of complex signalling pathways that are activated by the direct interaction of pattern recognition receptors (PRRs) and pathogen-associated molecular patterns (PAMPs) (Mogensen 2009). This is a key innate immune strategy against clearance of *Candida* infections. However, evidence shows that the complement system is essential for optimum resistance (Dunkelberger *et al.,* 2010).

C. albicans can activate the alternative complement system to promote opsonophagocytosis (Harpf *et al.,* 2020). There have been reports of *C. albicans* evading opsonophagocytosis through inhibition of complement activation (Harpf *et al.,* 2020). Nevertheless, the role of host environments in influencing complement inactivation has not been explored. This chapter aims at investigating on whether the currently reported oestrogen-dependent inhibition of phagocytosis occurred through inactivation of the complement system.

5.2 Results

5.2.1 Oestrogen-adapted *C. albicans* cells evade innate immunity by inhibiting opsonophagocytosis.

To study if the oestrogen derived immune evasion was due to complement inactivation, oestrogen-adapted *C. albicans* cells were incubated with macrophages previously maintained in media supplemented with heat inactivated serum. Customarily, media for macrophages is supplemented with live serum which among other things contain complement proteins such as C3 or C3b. Thus, use of macrophages maintained in media complemented with heat inactivated serum deprived the experimental set up with some proteins including complement proteins since heating at 56ºC denatures complement proteins. Largely, the uptake of *C. albicans* cells in an experiment set up lacking some proteins had lower phagocytosis rates compared to those in a complete set up (Figure 5.1A-B). This confirmed that some of the denatured proteins were necessary in promoting phagocytosis. Remarkably, there was no attenuation of phagocytosis for oestrogen-adapted *C. albicans* cells in an assay set up devoid of some proteins (Figure 5.1A-B). To probe further on whether the lack of complement proteins in the assay could necessitate loss of the phenotype, purified C3 or C3b proteins were supplemented to the experimental set up with denatured proteins. Interestingly, this led to restoration of the oestrogen-driven innate immune evasion (Figure 5.1A-B). This outcome suggested that inhibition of phagocytosis by oestrogen-adapted *C. albicans* cells was most probably due to evasion of complement opsonisation.

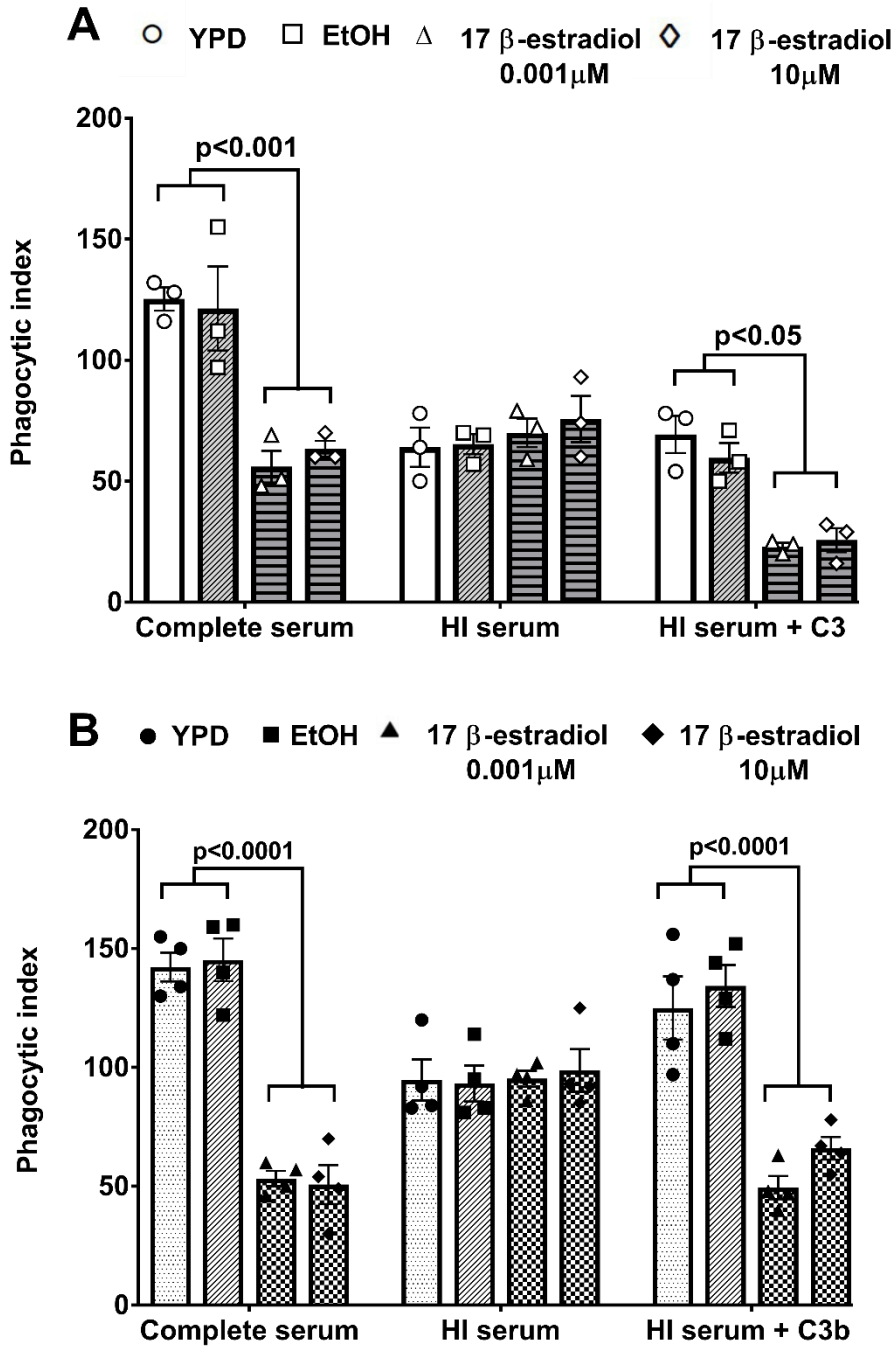

Figure 5.1. Oestrogen adapted *C. albicans* cells evade innate immune evasion through attenuation of opsonophagocytosis. J774A.1 cells were maintained in media complemented with either complete serum, heat-inactivated (HI) serum, or HI serum supplemented with purified (**A**) C3 (2.5 µg/mL) or (**B**) C3b (1 µg/mL) and infected with *C. albicans* at a MOI of 5. Phagocytic index data represent the mean ± SEM from at least three independent experiments. Data was analysed by Two-way ANOVA and Tukey's multiple comparisons test.

The discovery that adaptation to oestrogen promoted *C. albicans* immune evasion via avoidance of opsonophagocytosis suggested the process involved manipulation of the deposition of the key opsonins on the fungal cell surface. Therefore, the deposition of C3 and C3b on *C. albicans* post oestrogen treatment was evaluated. Oestrogen-adapted cells displayed enhanced C3 and C3b binding than control cells (Figure 5.2). Increased deposition of C3b or C3 onto fungal surface is generally associated with enhanced phagocytosis. It is therefore surprising to observe attenuation of phagocytosis despite more C3b or C3 deposited on oestrogen adapted *C. albicans.*

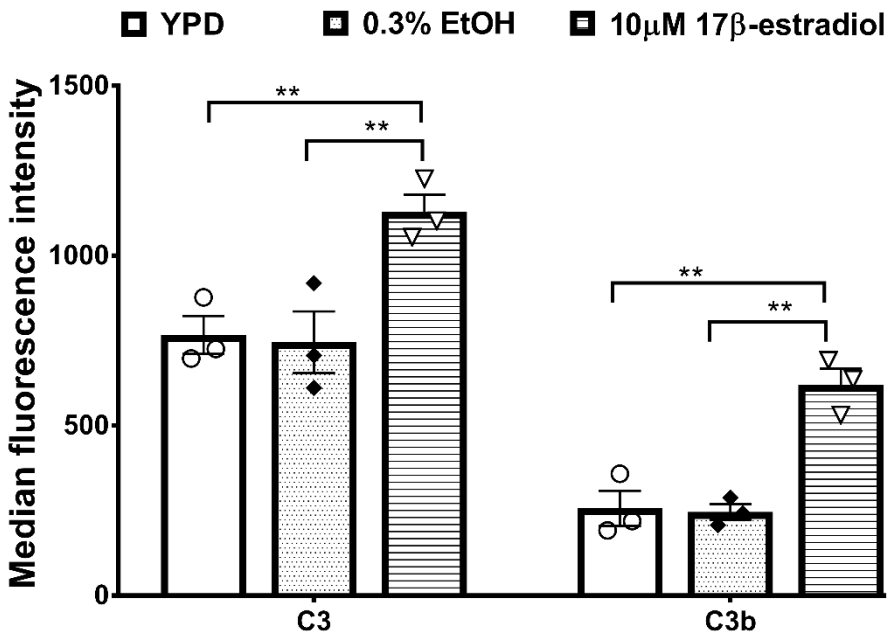

Figure 5.2. Adaptation to oestrogen promotes *C. albicans* binding to C3 and C3b. *C. albicans* was grown for 4 h in YPD with or without 17β-oestradiol. Cells were harvested, washed in PBS, and incubated in human serum for 20 min. Cells were washed and stained for C3 and C3b. Protein binding was quantified by FACS. MFI data represent the mean ± SEM from at least three independent experiments. Data was analysed by Two-way ANOVA and Tukey's multiple comparisons test; **p<0.01.

5.2.2 Adaptation of *C. albicans* to oestrogen enhance recruitment of Factor H.

Factor H (FH) is a negative regulator of the alternative pathway and the amplification loop of the complement cascade (Kopp *et al.,* 2012, Parente *et al.,* 2017). Furthermore, FH has the ability to recognise and bind to host surfaces in order to guard them from attack by the complement (Ferreira *et al.,* 2006). Interestingly, several bacterial and fungal pathogens have evolved to utilize this strategy and bind FH onto their surfaces and evade complement attack (Józsi 2017). Therefore, based on this background, an experiment was performed to assess the capability of oestrogen-adapted cells to bind to FH. Oestrogen-adapted *C. albicans* cells were treated with human serum and analysed for FH binding by FACS. Findings showed that adaptation of *C. albicans* to oestrogen to promoted binding of FH (Figure 5.3). Therefore, considering that FH is a complement inhibitor, this finding confirmed that adaptation of *C. albicans* to oestrogen promotes immune evasion through inhibition of opsonophagocytosis.

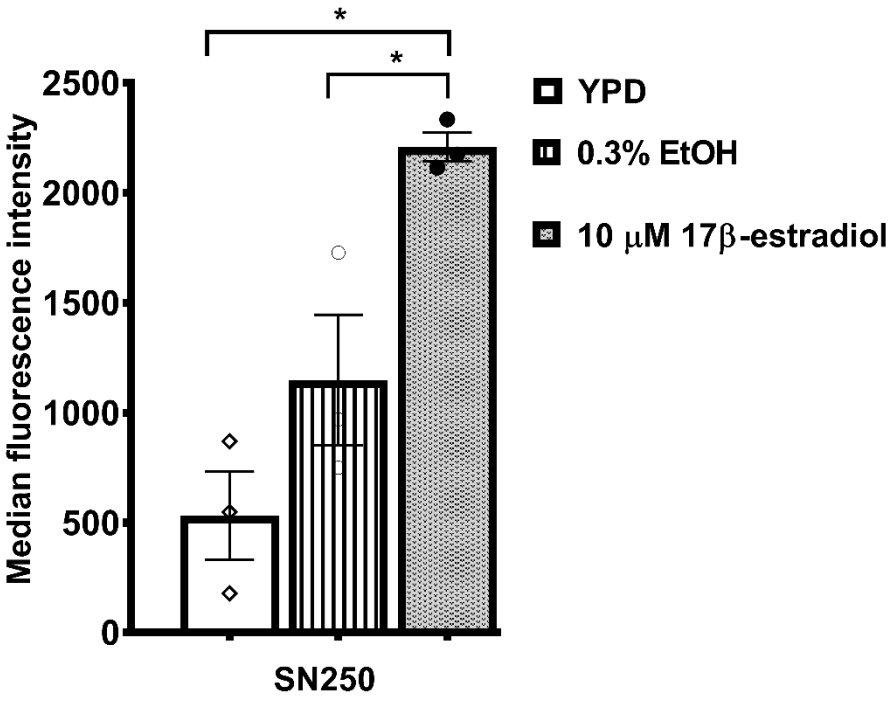

Figure 5.3. Adaptation of *C. albicans* to oestrogen enhances Factor H binding. *C. albicans* cells SN250 were grown for 4 h in YPD with or without 10 μM 17β-estradiol. Cells were harvested, incubated in human serum for 20 minutes, washed in PBS and stained for Factor H binding. Staining was quantified by flow cytometry and MFI determined. All data represent the mean ± SEM from at least three independent experiments. Data was analysed by Kruskal-Wallis test and Dunn's multiple comparisons test; *p<0.05.

5.2.3 The oestrogen-driven innate immune evasion is facilitated by Gpd2.

Besides the glycerol-3-phosphate dehydrogenase activity in the cytoplasm, Gpd2 also acts as a moonlighting protein (Luo *et al.,* 2013). A moonlighting protein is a protein that exhibits biological functions unpredicted by its amino acid sequence (Jeffery 2018). Some of the moonlighting activities of Gpd2 include binding FH and plasminogen which are essential in regulating the alternative complement cascade (Luo *et al.,* 2013).

Recent studies have also revealed that in response to serum, *C. albicans* Gpd2 can localise to the cell surface (Marin *et al.,* 2015). Data from earlier experiments in the present study showed that *C. albicans* glycerol-3-phosphate dehydrogenase 2 (Gpd2) is important in mediating the oestrogen-dependent innate immune evasion. In addition, RNA sequencing data of oestrogen adapted *C. albicans* cells revealed that *GPD2* is one the differentially upregulated genes in response to oestrogen. Based on this background and the fact that oestrogen adapted *Candida* cells bound more FH, an investigation was carried out to determine whether the increase in FH recruitment was facilitated by Gpd2. To test this hypothesis, *C. albicans* cells (SN250 and *gpd2*Δ) were incubated in oestrogen and treated with serum for FH FACS analysis. Results indicated that there was less FH binding in *gpd2*Δ as compared to the wild type (Figure 5.4). Therefore, Gpd2 facilitates the oestrogen-dependent innate immune escape by increasing the recruitment of FH which inhibits the complement mediated opsonophagocytosis.

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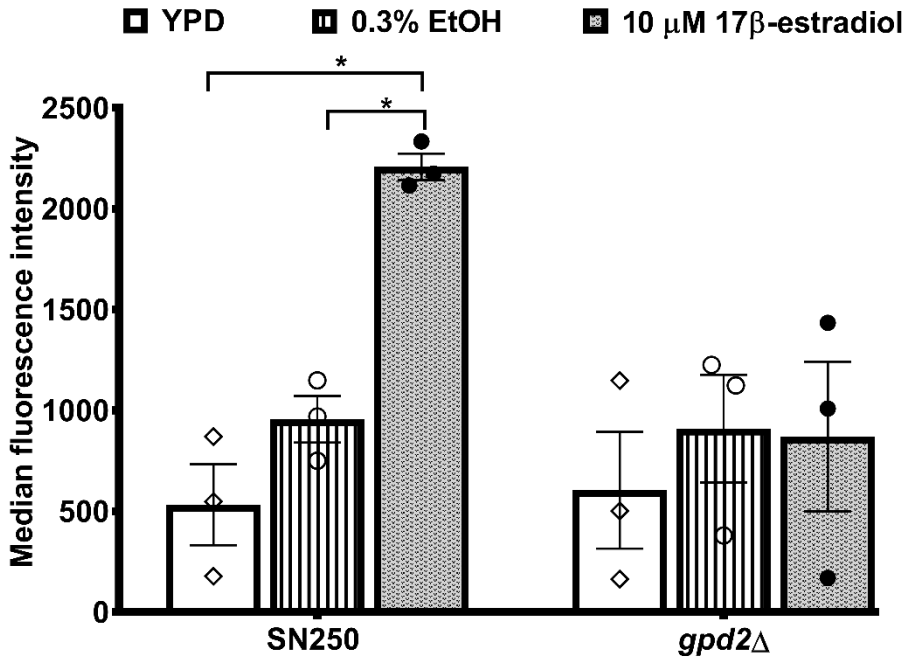

Figure 5.4. Gpd2 facilitates the oestrogen-driven innate immune escape. *C. albicans* strains SN250 and *gpd2*Δ were separately grown for 4 h in YPD with or without 10 μM 17β-oestradiol. Cells were harvested, washed with PBS, and then incubated in human serum for 20 minutes, fixed with PFA and stained for Factor H binding. Factor H binding was quantified by FACS. All data represent the mean ± SEM from at least three independent experiments. Data were analysed by Two-way ANOVA and Tukey's multiple comparisons test; **p < 0.01.

Gpd2 is known to bind to plasminogen (Luo *et al.,* 2013), a complement inhibitor (Barthel *et al.,* 2012). Thus, a hypothesis that plasminogen could inhibit opsonophagocytosis was formulated. To test this hypothesis, macrophages maintained in media supplemented with heat inactivated serum were complemented with plasminogen and C3b proteins and incubated with oestrogen-adapted *C. albicans* cells. Generally, supplementation of C3b improved phagocytosis for the YPD and ethanol controls (Figure 5.5). Plasminogen alone had no impact on phagocytosis, but significant inhibition of phagocytosis was observed when both plasminogen and C3b were added (Figure 5.5).

This outcome suggested that plasminogen inhibited or cleaved C3b thereby supressing opsonophagocytosis. Therefore, plasminogen is also important in promoting the oestrogen dependent innate immune evasion.

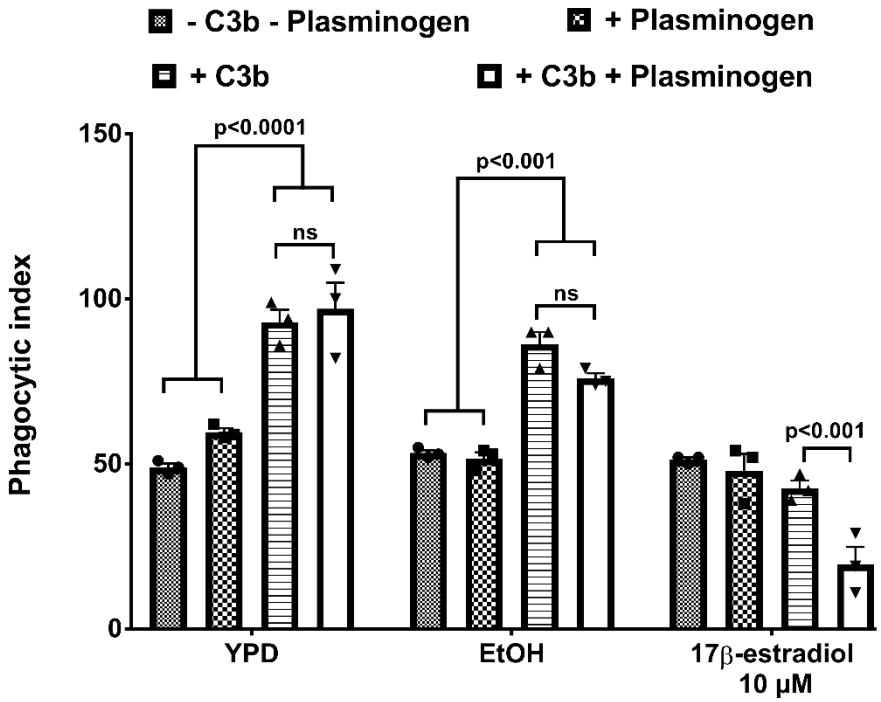

Figure 5.5. Plasminogen plays a role in promoting oestrogen-mediated innate immune evasion. J774A.1 cells were maintained in media prepared with heat-inactivated (HI) serum, or HI serum supplemented with purified C3b, plasminogen or C3b and plasminogen respectively. Macrophages were infected with control and oestrogen adapted *C. albicans* cells at a MOI of 5. Phagocytic index data represent the mean ± SEM from at least three independent experiments. Data were analysed by Two-way ANOVA and Tukey's multiple comparisons test; ***p<0.001.

Altogether this data suggest that Gpd2 is essential in mediating the oestrogen-dependent innate immune evasion by enhancing binding of Factor H, a complement regulatory protein critical in inhibiting opsonophagocytosis. In addition, plasminogen also seems to play a significant role in facilitating the oestrogen-driven innate immune evasion by inactivating the activity of C3b.

5.3 Discussion

There are several ways *C. albicans* can be detected by the innate immune system. The complement system is one of the natural immune strategies that recognises the fungus as foreign and targets it for destruction. The complement system can be activated by fungal cell wall components such as mannose (Van Asbeck *et al.,* 2008), chitin (Roy *et al.,* 2013) and β-glucan (Boxx *et al.,* 2010). Notably, activation of complement cascades generates opsonins which, when deposited on *C. albicans,* promote opsonophagocytosis (Van Asbeck *et al.,* 2008). Thus, stimulation of the complement system by host environments promotes opsonophagocytosis. However, *C. albicans* has devised mechanisms to escape the anti-*Candida* effects of the complement system.

Different microbes including *C. albicans* have been reported to evade the complement system. Microbes evade the complement effector functions by controlling the activities of the cascade via recruitment of the complement regulatory proteins onto their surface and or by proteolytically degrading the complement proteins (Meri *et al.,* 2002, Meri *et al.,* 2004, Gropp *et al.,* 2009, Losse *et al.,* 2010, Luo *et al.,* 2013). FH, FH-L, plasminogen and C4BP are examples of the complement regulatory proteins recruited by various microbes. Whilst bound to pathogens, the complement regulatory proteins are active hence the microbes regulate their functions to prevent attack (Zipfel *et al.,* 1999, Meri *et al.,* 2002, Meri *et al.,* 2004, Zipfel *et al.,* 2007, Behnsen *et al.,* 2008, Zipfel *et al.,* 2008, Agarwal *et al.,* 2010).

Gpd2 is one of the cell surface moonlighting proteins identified in *C. albicans* to bind to FH, FH-L and plasminogen. Luo and colleagues demonstrated that *C. albicans* Gpd2 binds to FH and FH-L and inhibit complement activation (Luo *et al.,* 2013). Investigation in the current study revealed that following adaptation to oestrogen, the wild type strain bound more Factor H than the *gpd2* mutant thereby confirming that the expression of Gpd2 plays a key role in regulating the oestrogen driven immune evasion.

C. albicans Gpd2 is predicted as a cytoplasmic protein participating in redox reactions, however, Marin *et al*. (2015) has demonstrated that the protein is also present in cell wall proteome of the fungus. This suggests that Gpd2 also carries out other function in the cell wall. In addition, *C. albicans* expresses Gpd2 in the cell wall when incubated in complete serum but not in heat inactivated serum which lacks some active complement proteins (Marin *et al.,* 2015). Therefore, deposition of complement proteins on *C. albicans* cell surface may promote cell surface localisation of the Gpd2 protein.

FH is one of the complement regulators that inactivates the alternative pathway and the complement amplification loop (Hammel *et al.,* 2007, Parente *et al.,* 2017). FH achieves this by binding to C3b and inhibit formation of C3 convertase (C3bBb) as well as by inhibiting the activity of the C5 convertase (Józsi 2017). FH also facilitates the decay of previously generated C3bBb by detaching the Bb from C3b.

In addition, FH functions as a cofactor for Factor I, a complement inactivator that proteolytically cleaves C3b and C4b complement proteins (Józsi 2017). The present study revealed that complement deposition on oestrogen adapted *C. albicans* cells enhances FH binding than on wild type cells, thus signifying that oestrogen promote evasion of complement.

Additionally, adaptation of *C*. *albicans* to oestrogen led to increased C3b binding. This is an unexpected outcome since increased binding of FH should subsequently enhance C3b degradation and therefore less deposition of the complement on *C. albicans* cell surface. FH is comprised of 20 complement control proteins (CCP) to which microbes are known to bind (Kristensen *et al.,* 1986, Ripoche *et al.,* 1988). CCP20 is the common FH binding site for several microbes (Meri *et al.,* 2013). The recruitment of FH on microbial cell surface through CCP20 binding increases the affinity of FH for C3b (Meri *et al.,* 2013). Thus, the increased affinity of FH for C3b might provide a clue on why there was enhanced binding of C3b and FH. Furthermore, this study has shown that adaptation to oestrogen promoted more FH binding in wild type strain than *gpd2*∆. Therefore, this would imply that Gpd2 binds FH via CCP20.

Staphylococcus aureus has been reported to use Efb-C protein (*S. aureus* extracellular fibrinogen-binding protein) to inhibit complement activation (Hammel *et al.,* 2007). Efb-C binds to C3 and change its conformation to one that cannot be processed to C3b, a vital complement protein necessary for the assembly of the C3 convertase (Hammel *et al.,* 2007).

Efb-C has also been shown to bind to C3b containing convertases (i.e. C3 convertase and C5 convertase) and block complement stimulation (Jongerius *et al.,* 2007). Therefore, the increased binding of C3 and subsequent reduction in phagocytosis of oestrogen adapted *Candida* cells might be associated with Gpd2 binding to C3 and changing its conformational structure to render the complement protein incapable of participating in the successful downstream activation of the complement cascade. As a result, this process may increase bound C3 and reduce generation of C3b thus promoting inhibition of opsonophagocytosis. This is an interesting theory that requires further investigation to demonstrate whether Gpd2 has properties to manipulate the structure of C3 or bind to C3b containing convertases and inhibit complement activation.

Similar to most microbial FH binding proteins (Kunert *et al.,* 2007, Poltermann *et al.,* 2007, Grosskinsky *et al.,* 2009), Gpd2 is also able to bind plasminogen (Luo *et al.,* 2013). Plasminogen promotes Factor I-mediated C3b degradation in the presence of FH (Barthel *et al.,* 2012). Plasmin, the proteolytically active form of plasminogen also cleaves and inactivates C3b and C5 (Barthel *et al.,* 2012). Overall, these activities inhibit complement activation and progression (Barthel *et al.,* 2012). As demonstrated in this study, *C. albicans* adaptation to oestrogen enhanced expression of *GPD2* and supplementation of C3b and plasminogen to a phagocytosis assay promoted inhibition of phagocytosis of oestrogen adapted *C*. *albicans* cells.

Therefore, this suggest that *C. albicans* adaptation to oestrogen may enhance binding of plasminogen which subsequently degrades complement and promote inhibition of opsonophagocytosis.

5.4 Conclusion

The complement system is part of innate immunity and it provides defence against *C. albicans* by enhancing phagocytosis through opsonisation of the fungus (Harpf *et al.,* 2020). Nevertheless, *C. albicans* evades the complement system by recruiting negative regulators of the complement on its surface, masking PAMPs that trigger the complement cascade and production of aspartyl proteases that degrade complement proteins (Harpf *et al.,* 2020). The current study has shown that the complement system is an essential target of the oestrogen depended innate immune evasion by *C. albicans.*

6 The role of oestrogen on *C. albicans* pathogenicity

6.1 Background

Currently there are several vertebrate and invertebrate infection model systems used to study fungal pathogenicity (Arvanitis *et al.,* 2013, Hohl 2014). This study utilised *Galleria mellonella* and Zebrafish larvae infection models to study the role of oestrogen on *C. albicans* pathogenicity *in vivo*.

6.1.1 Zebrafish (*Danio rerio*) infection model

Recent developments indicate that zebrafish (*Danio rerio*) are an attractive model system to study host-pathogen interactions for bacterial and fungal pathogens (Torraca *et al.,* 2014, Mallick *et al.,* 2016). Models using embryos, larvae or the adult zebrafish have been established to investigate mucosal and disseminated *C. albicans* infections (Brothers *et al.,* 2012, Gratacap *et al.,* 2014, Chen *et al.,* 2015). The zebrafish models provide a temperature-dependent system for investigations since its growth temperature range span from 23 to 33°C (López-Olmeda *et al.,* 2011).

Furthermore, zebrafish provide a greater advantage for conducting host-pathogen interaction studies to other non-mammalian models (i.e. *G. mellonella)* because it has an innate immune system comprising of neutrophils (Le Guyader *et al.,* 2008), macrophages (Herbomel *et al.,* 1999), and complement (Seeger *et al.,* 1996).

Moreover, the innate immune system of zebrafish larvae is highly similar to the one in humans thus enhancing opportunities for understanding host-pathogen interactions studies (Novoa *et al.,* 2012, van der Vaart *et al.,* 2012). In addition, the complement system in zebrafish is similar to the one in humans with all orthologues of mammalian complement components identified in zebrafish (Zhang *et al.,* 2014). Interestingly, the adaptive immune system in zebrafish develops later than innate immunity (i.e. 2-3 weeks after fertilisation) (Willett *et al.,* 1999, Lam *et al.,* 2004). Therefore studies targeting the innate immune system can be conducted without interference of adaptive immunity. Other experimental benefits of zebrafish include their rapid development thereby permitting results to be achieved quicker, optical transparency thus allowing direct, real-time non-invasive imaging of the host and microbial cells throughout the experimental process (Meeker *et al.,* 2008, Knox *et al.,* 2014) (Figure 6.1). In addition, zebrafish larvae can be injected/infected at different sites depending at the type of investigation to be carried out (Figure 6.1).

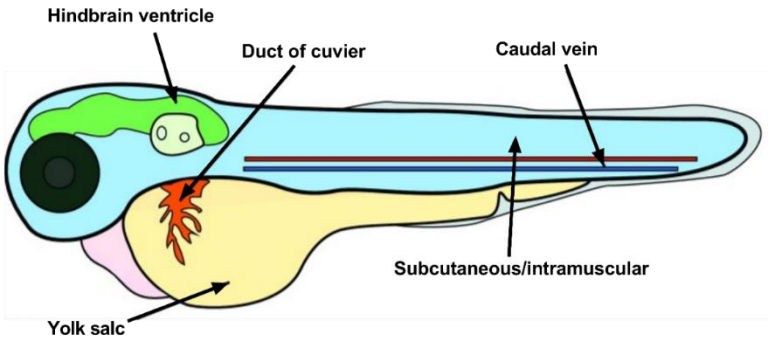

Figure 6.1. Common injection sites in zebrafish larvae infection model. The figure indicates different injection sites used to achieve specific study objectives. In the present study, hindbrain infection was performed to achieve *C. albicans* disseminated infection. Image was copied from Veinotte *et al*., (2014).

Oestrogen is vital in reproductive biology of vertebrates including fish (Amenyogbe *et al.,* 2020). Oestrogen receptors Esr1, Esr2a and Esr2b have been characterised in zebrafish (Bardet *et al.,* 2002). However, the zebrafish larvae must be treated with supraphysiological levels of oestrogen to achieve physiologically relevant concentration *in vivo* (Souder *et al.,* 2017). Thus, this scenario provides an opportunity to study the impact of oestrogen on host pathogen interaction *in vivo*.

6.1.2 Zebrafish (*Danio rerio*) infection model

Galleria mellonella larva are widely used as an experimental model to investigate host–pathogen interactions and the efficacy of antimicrobial agents in bacterial and fungal pathogens (Sheehan *et al.,* 2018, Cutuli *et al.,* 2019) (Figure 6.2).

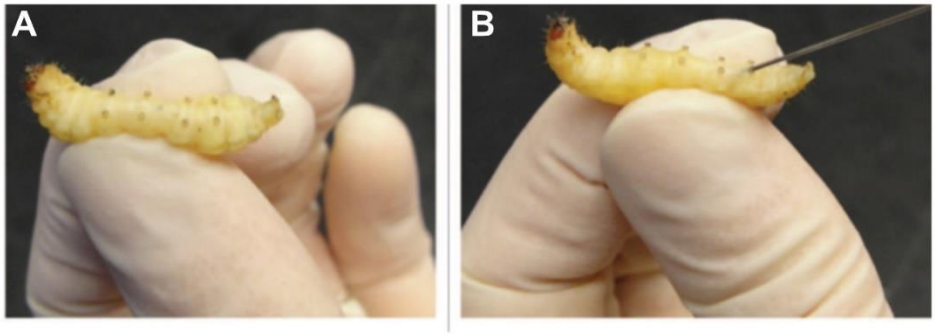

Figure 6.2. *G. mellonella* larvae. (**A**) To avoid larva movement and allow stability, a slight amount of pressure is applied while holding the larvae between fingers (**B**) To inoculate microbe under study, larvae are held between fingers as described above thereby exposing the pro-legs. With a slight amount of pressure applied to the larva body, a needle is inserted at the site of the pro-leg and inject the required volume of the organism. Image was copied from (*Fuchs et al*., 2010)

There are several advantages for using *G. mellonella* larva model. In comparison to vertebrate models, *G. mellonella* is cheaper and easier to maintain. The larvae are large enough for easy handling and extraction of tissue for further analysis. The larger size of the larvae enhances the precise inoculation of the right quantity of the pathogen under study (Cook *et al.,* 2013). The short life cycle of the larva makes it appropriate for high-throughput investigations (Tsai *et al.,* 2016, Sheehan *et al.,* 2018). Additionally, the larva has some physiological and immunological features that makes it a successful model. For example, *G. mellonella* larvae can be maintained at a mammalian body temperature of 37^{o}C. This characteristic is essential in studying the virulence of pathogens since temperature is known to influence the expression of virulence factors in microbes (Fuchs *et al.,* 2010, Tsai *et al.,* 2016, Twittenhoff *et al.,* 2020). *G. mellonella* lacks an adaptive immune system but its innate immune system shares a lot of resemblance with the vertebrate innate immunity (Sheehan *et al.,* 2018). Consequently, the larvae can be utilised to carry out host pathogen interaction studies with particular focus on innate immune response.

Previous experiments in this study revealed that oestrogen induces expression of Gpd2 which then inactivates complement promoting *C. albicans* innate immune evasion. Therefore, this chapter sought to investigate whether this phenomenon could have an impact on *C. albicans* virulence *in vivo* using the *Galleria mellonella* and zebrafish larvae infection models.

6.2 Results

6.2.1 Oestrogen promotes *C. albicans* virulence in zebrafish larvae

The present study has revealed that adaptation to oestrogen promotes innate immune evasion via inhibition of complement mediated opsonophagocytosis. Since zebrafish has a complement system highly comparable to humans, a zebrafish larvae infection model was created to examine whether adaptation to oestrogen can influence fungal virulence *in vivo.* Oestrogen can diffuse into zebrafish larvae once incubated in water or media containing the hormone (Souder *et al.,* 2017). Therefore, the larvae were inoculated with *C. albicans* and maintained in E3 media or E3 media containing either 0.3% ethanol or 10 μM 17β-oestradiol. Larvae were observed for up to 5 days post fertilisation and their survival rates were 38%, 47% and 13% respectively (Figure 6.3). Thus, a significant drop in survival of zebrafish was observed in oestrogen complemented media in comparison to the ethanol and standard media controls ($p<0.001$, $p<0.05$ respectively).

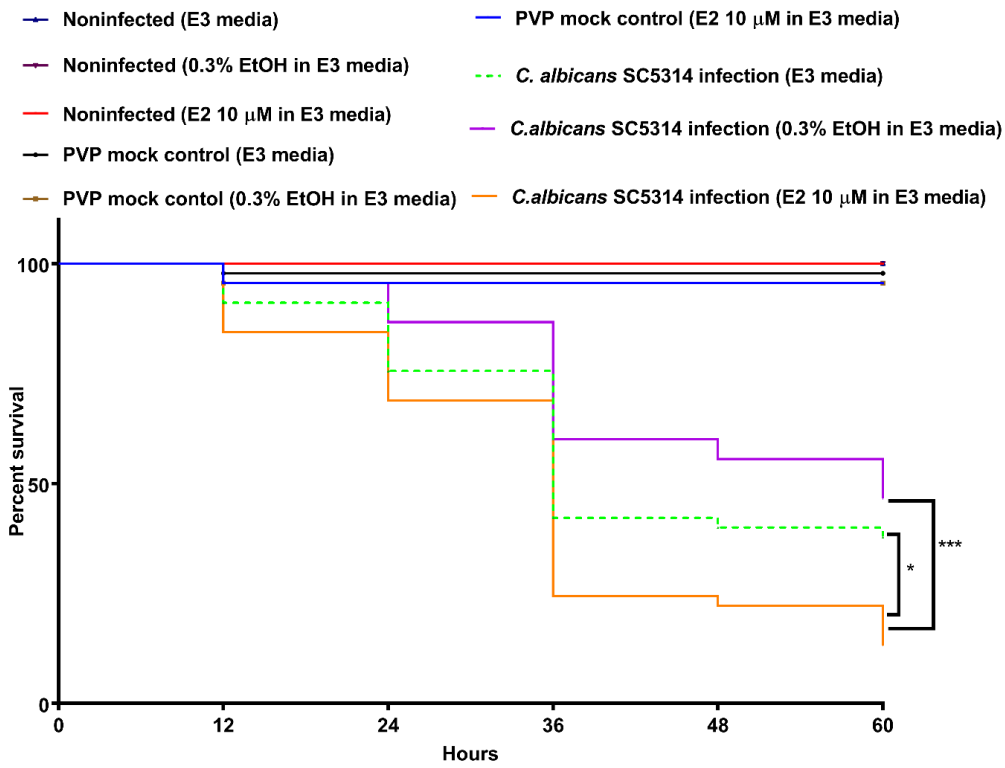

Figure 6.3. Oestrogen promotes the virulence of *C. albicans* in a zebrafish larval infection model. *C. albicans* (SC5314) cells were microinjected into the hindbrain ventricle of 20 zebrafish larvae in the Prim25 stage. Infected larvae were maintained in E3 media, or E3 media supplemented with 0.3% ethanol, or 10 μM 17β-oestradiol (E2). Larval survival was monitored every 24 h up to day 5 post fertilisation. The survival curves represent data pooled from three independent experiments. Statistically significant differences were determined by Log-rank (Mantel-Cox) test; *p<0.05, ***p<0.001.

Previous investigations have demonstrated that exposure of zebrafish to 1 μM oestrogen for 4 days from 3 h post fertilisation results in *in vivo* oestrogen levels of about 0.057 μM, a concentration comparable to physiological levels of the hormone during pregnancy in humans (Hao *et al.,* 2013, Souder *et al.,* 2017). Thus, zebrafish larvae were incubated in E3 media supplemented with 1 μM oestrogen.

Similarly, there was a significant drop in survival of zebrafish larvae incubated in oestrogen supplemented media in comparison to ethanol and standard media controls ($p<0.05$) (Figure 6.4). The survival rates of zebrafish were 41% in standard E3 media, 35% in ethanol supplemented E3 media and 19% for those incubated in E3 media complemented with 1 µM oestrogen. Altogether, this suggest that oestrogen is essential in promoting *C. albicans* virulence *in vivo*.

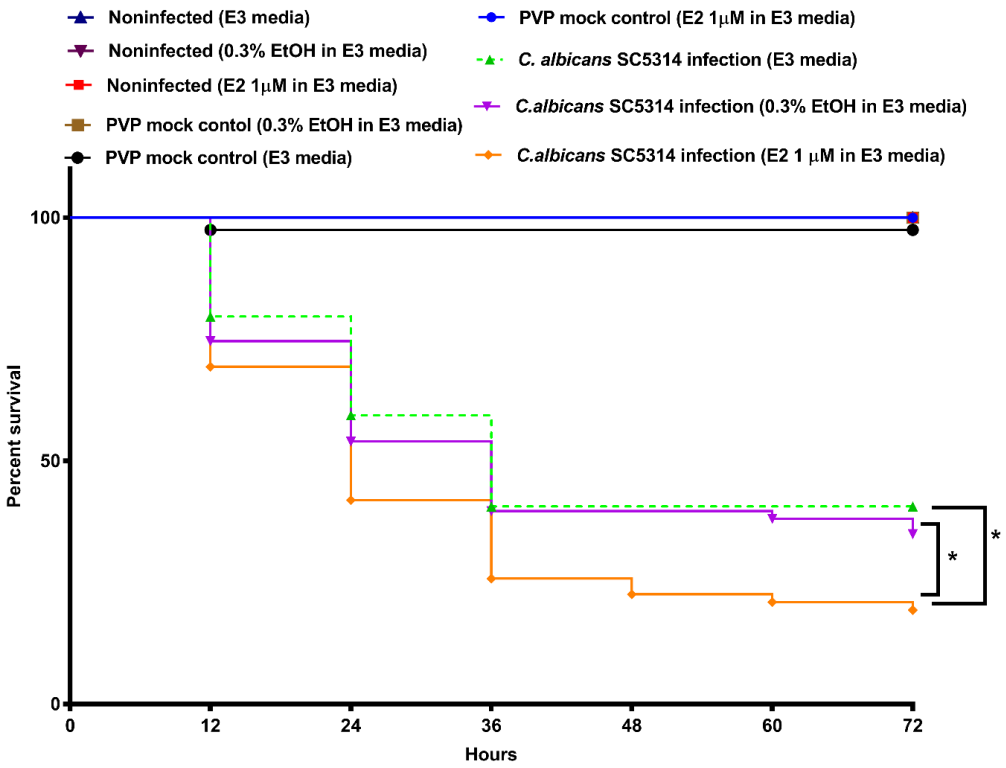

Figure 6.4. Oestrogen promotes the virulence of *C. albicans* in a zebrafish larval infection model. *C. albicans* (SC5314) cells were microinjected into the hindbrain ventricle of 20 zebrafish larvae in the Prim25 stage. Infected larvae were maintained in E3 media, or E3 media supplemented with 0.3% ethanol, or 1 µM 17β-oestradiol (E2). Larval survival was monitored every 24 h up to day 5 post fertilisation. The survival curves represent data pooled from three independent experiments. Statistically significant differences were determined by Log-rank (Mantel-Cox) test; *$p < 0.05$.

6.2.2 Oestrogen does not promotes *C. albicans* virulence in *Galleria mellonella* larvae

Considering that *G. mellonella* lacks complement system analogous to humans, an investigation was carried out to verify the importance of complement in the oestrogen mediated fungal immune evasion. *G. mellonella* larvae infection model was used to study disease progression following infection with *C. albicans*.

G. mellonella larvae were infected with wild type *C. albicans* cells and *C. albicans* cells adapted to either 0.001 µM or 10 µM oestrogen. In addition, the larvae were primed with either 0.001 µM or 10 µM oestrogen prior to infection with wild type *C. albicans* cells. Survival rates of the larvae were monitored for 96 h. Results showed that adaptation of *C. albicans* to oestrogen did not influence virulence of the fungus *in vivo.* The survival rates of test and control larvae were similar with no statistically significant differences ($p>0.05$) i.e., 26% (wild type control), 13% (10 µM oestrogen adapted *C. albicans* cells) and 17% (10 µM oestrogen primed larvae) for Figure 6.5 and 26% (wild type control), 20% (0.001 µM oestrogen adapted *C. albicans* cells) and 13% (0.001 µM oestrogen primed larvae) for Figure 6.6.

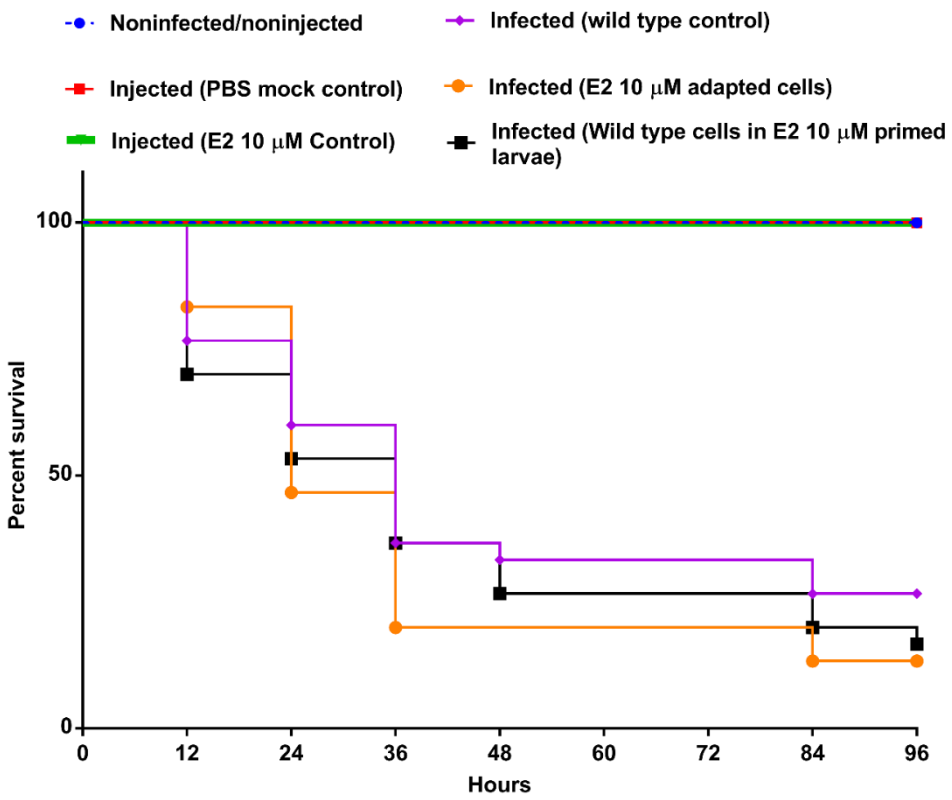

Figure 6.5. Oestrogen does not promote *C. albicans* virulence in *G. mellonella* larvae. *G. mellonella* larvae were either infected with wild type and oestrogen adapted *C. albicans* cells or primed with 10 μM oestrogen and infected with wild type *C. albicans* cells. Larva were monitored for 96 h and percent survival were determined. The figure represents pooled data from three biological repeats with 10 larvae per experimental condition. A Log-rank (Mantel-Cox) test statistical analysis was performed using Graph pad prism 7.

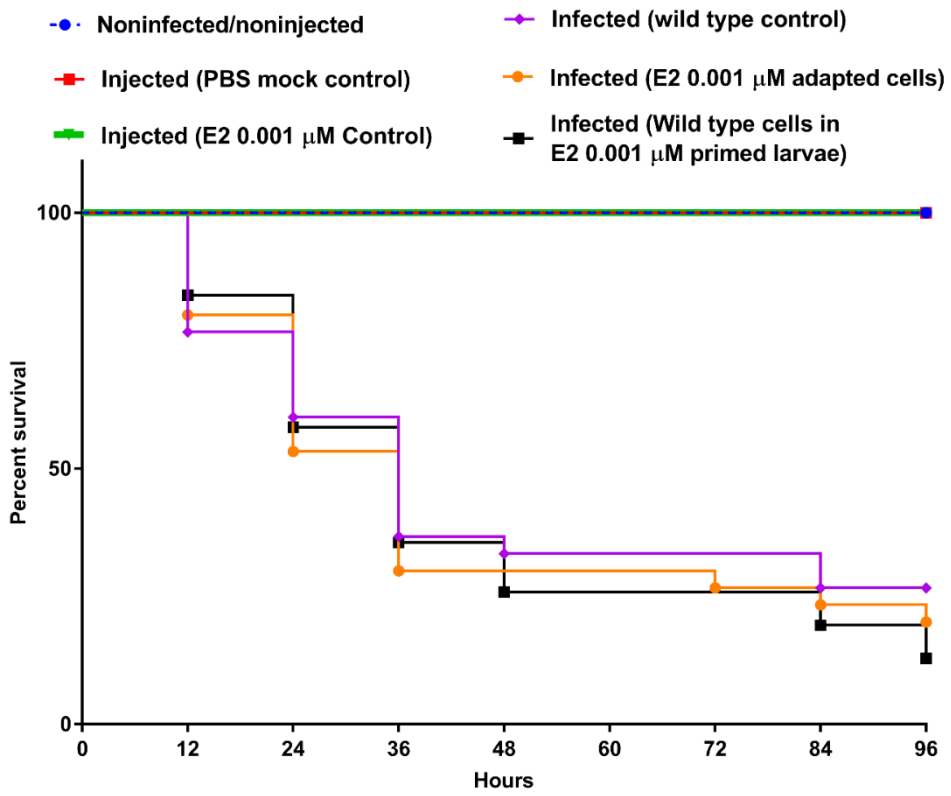

Figure 6.6. Oestrogen does not promote *C. albicans* virulence in *G. mellonella* larvae. *G. mellonella* larvae were either infected with wild type and oestrogen adapted *C. albicans* cells or primed with 0.001 μM oestrogen and infected with wild type *C. albicans* cells. Larva were monitored for 96 h and percent survival were determined. The figure represents pooled data from three biological repeats with 10 larvae per experimental condition. A Log-rank (Mantel-Cox) test statistical analysis was performed.

6.2.3 Gpd2 is linked with the oestrogen-dependent *C. albicans* virulence *in vivo*

Findings from the present study reveal that Gpd2 is necessary for oestrogen-dependent innate immune escape mechanism. Therefore, to investigate whether Gpd2 is essential for virulence *in vivo*, zebrafish larvae were microinjected with *C. albicans* SN250-CIp30, *C. albicans gpd2*Δ-*CIp30* and *C. albicans gpd2*Δ-CIp30-*GPD2* (a Gpd2 reconstituted strain).

Loss of *GPD2* led to attenuation of *C. albicans* virulence as demonstrated by a higher zebrafish survival rate (i.e., 54%) in comparison to the parental control strain SN250-CIp30 (survival rate 33%, $p<0.05$) (Figure 6.7). In addition, reconstitution of *GPD2* in the mutant led to restoration of virulence with the survival rate of zebrafish dropping to 23% ($p<0.01$) (Figure 6.7). Thus, this outcome suggest that Gpd2 is important promoting *C. albicans* virulence *in vivo*.

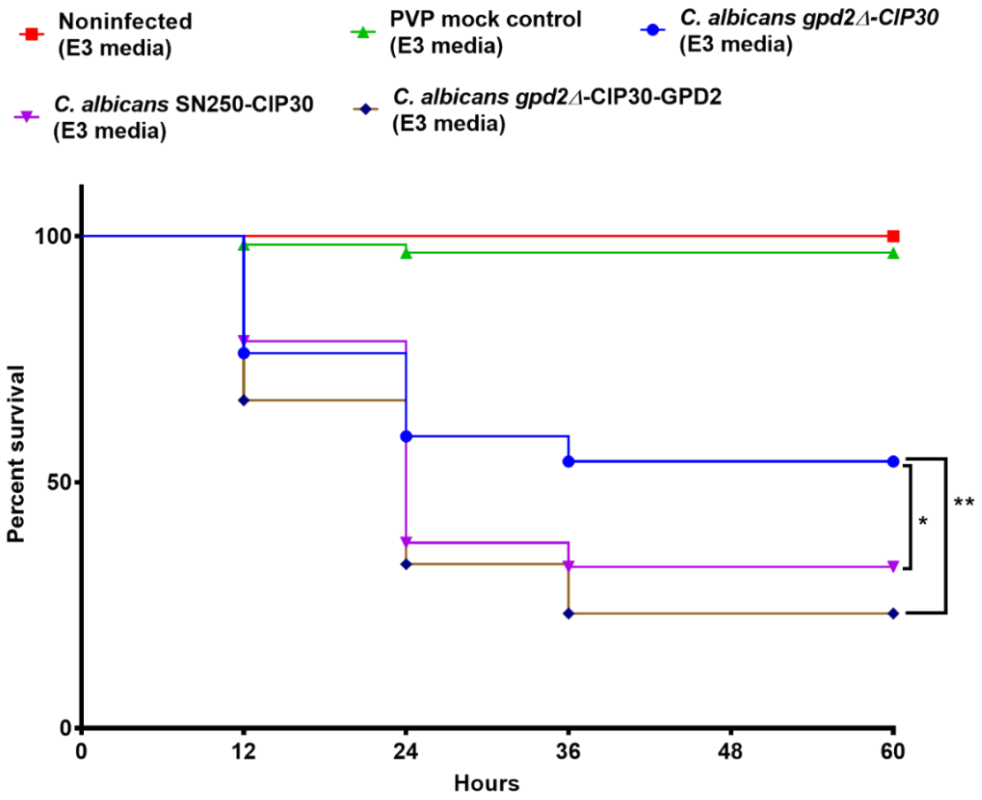

Figure 6.7. Deletion of GPD2 leads to loss of virulence of *C. albicans* in a zebrafish larval infection model. *C. albicans* SN250-CIp30, gpd2Δ-CIp30 and gpd2Δ-CIp30-GPD2 were microinjected into the hindbrain ventricle of 20 zebrafish larvae in the Prim25 stage dpf. Larvae were maintained in E3 media and larval survival monitored every 24 h up to day 5 post fertilisation. The survival curves represent data pooled from three independent experiments. Statistically significant differences were determined by Log-rank (Mantel-Cox) test; *$p<0.05$, **$p<0.01$.

To validate the role of Gpd2 in promoting *C. albicans* virulence, *GPD2* was overexpressed in the *C. albicans* CAI4 background and zebrafish larvae infected with the strain to examine whether overexpression of the gene alone would promote virulence *in vivo*. Overexpression of *GPD2* led to heightened virulence in *C. albicans* as the survival rate of infected zebrafish reduced to 40% ($p<0.001$) unlike the parental control group which dropped to 73% (Figure 6.8). Therefore, this suggests that indeed Gpd2 plays a significant role in enhancing *C. albicans* virulence *in vivo*.

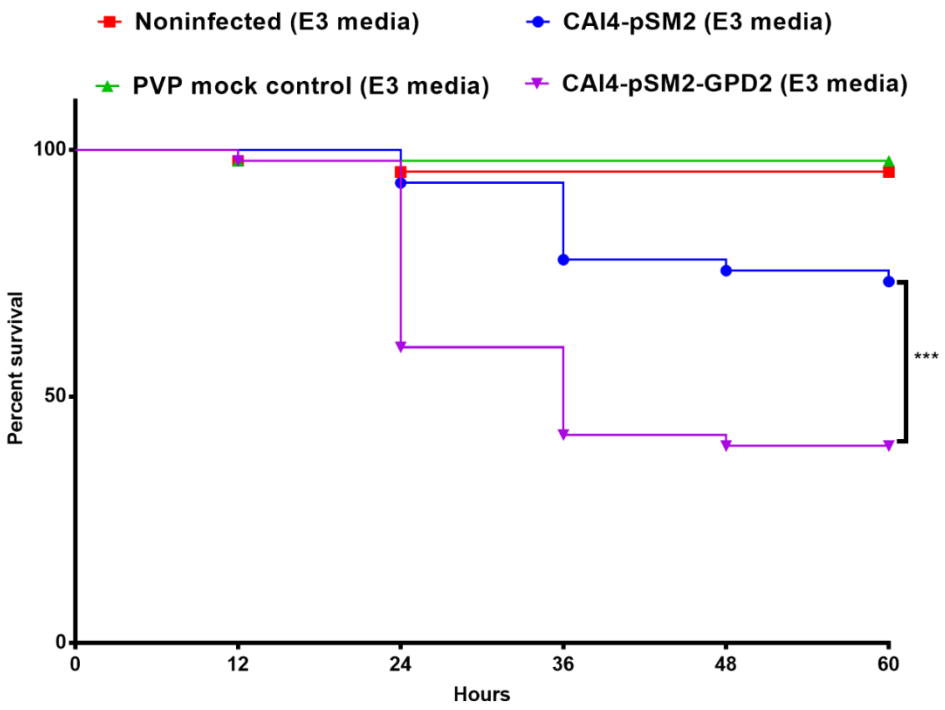

Figure 6.8. Overexpression of *GPD2* promotes the virulence of *C. albicans* in a zebrafish larval infection model. *C. albicans* CAI4-pSM2 or CAI4-pSM2-GPD2 were microinjected into the hindbrain ventricle of 20 zebrafish larvae in the Prim25 stage dpf. Infected larvae were maintained in E3 media. Larval survival was monitored every 24 h up to day 5 post fertilisation. The survival curves represent data pooled from three independent experiments. Statistically significant differences were determined by Log-rank (Mantel-Cox) test; ***$p<0.001$.

6.3 Discussion

Vaginal candidiasis (VVC) is a common infection in women of reproductive age group, and it is predominantly caused by *C. albicans*. As earlier discussed, oestrogen is one of the key predisposing factors for VVC. *In vitro* work in this study has demonstrated that adaptation of *C. albicans* to oestrogen promotes innate immune evasion. Nevertheless, the influence of oestrogen on *C. albicans* virulence *in vivo* has not been fully explored. Using a zebrafish infection model, this study has established that oestrogen promotes the virulence of *C. albicans in vivo* in a Gpd2 dependent manner. A disseminated zebrafish infection model was used since the current license could not permit us to do a zebrafish mucosal infection model.

The innate immune system in *G*. *mellonella* is comprised of the cellular and humoral immune response (Browne *et al.,* 2013). The cellular response is facilitated by haemocytes which play a similar function to phagocytes in humans (Wu *et al.,* 2016). The humoral response is characterised by the presence of soluble effector molecules including complement-like proteins (opsonins) (Sheehan *et al.,* 2018). *G. mellonella* does not contain a complement system like the one in mammals however like in humans, their opsonins are capable of detecting and binding to PAMPs such as lipoteichoic acid, lipopolysaccharides, and peptidoglycan thereby enhancing their innate immune response (Pereira *et al.,* 2020). Unlike *G. mellonella,* the complement system in zebrafish is quite similar to the one in mammals (Zhang *et al.,* 2014).

Previously published work and a search from zebrafish genome databases confirm that zebrafish contains all the homologs of complement components that have been identified in mammals (Zhang *et al.,* 2014). In addition, a phylogenetic analysis reveals bootstrap values of greater than 50% for most genes, with the value of Factor H showing as 49% (Zhang *et al.,* 2014). Previously described findings from the present study have shown that oestrogen adapted *C. albicans* cells evades immunity by inhibiting complement mediated opsonophagocytosis via Gpd2. Therefore, the presence of a vertebrate analogue complement system in zebrafish could be vital in promoting virulence of the fugus *in vivo*.

Previous investigations have shown that oestrogen promotes the progression of *C. albicans* infection *in vivo*. For instance, supplementation of oestrogen in ovariectomized mice leads to poor response to *C. albicans* systemic infection as shown by reduced survival of mice and enhanced fungal load in the tissue (Relloso *et al.,* 2012). Relloso *et al*., (2012) further revealed that supplementation of oestrogen weakens the host Th17 immune response against *C. albicans*. Th17 are vital in providing immune defence against *C. albicans* infection (Hernández-Santos *et al.,* 2012). Similarly, another study showed that treatment of gonadectomized male mice with oestrogen led to poor prognosis since mice lost weight and their rate of survival was reduced (Arroyo-Mendoza *et al.,* 2020). In addition, Arroyo-Mendoza *et al*., (2020) observed that oestrogen compromised the capability of dendritic cells to present *C. albicans* antigens to T lymphocytes thus inhibiting the process of polarisation of T cells to Th17 cell type.

Earlier studies also demonstrate that elevated oestrogen levels promote bias of immune defence from Th1 (IFNγ) to Th2 (IL-4) and manipulate the functions of Th17 and Treg cells thereby compromising immunity against *C. albicans*. Thus, in agreement with previous findings, the present study provides an additional theory that oestrogen may enhance *C. albicans* pathogenicity *in vivo* by promoting innate immune evasion through inhibition of complement.

6.4 Conclusion

Considering that oestrogen is a key risk factor for VVC, the discovery that the hormone can enhance *C. albicans* virulence *in vivo* and promote infection could improve our understanding on the role of oestrogen in influencing fungal infections *in vivo*. In addition, new ideas on preventive and therapeutic options for Candidiasis could be developed.

7 Discussion

7.1 Overview of key research finding

Vaginal candidiasis (VVC) is a common infection in women of reproductive age group, and it is predominantly caused by *C. albicans* (Sobel 2007). Much progress has been made in identifying host and fungal factors that promote VVC and oestrogen is one of the multiple factors that contribute to the development of VVC (Figure 7.1) (Willems *et al.,* 2020). Despite this, little has been done on elucidating how some host environments including oestrogen modulate the fungus to become more virulent and enhance VVC infection. This study employed *in vitro* and *in vivo* experiments to ascertain the impact of oestrogen on fungal host pathogen interaction.

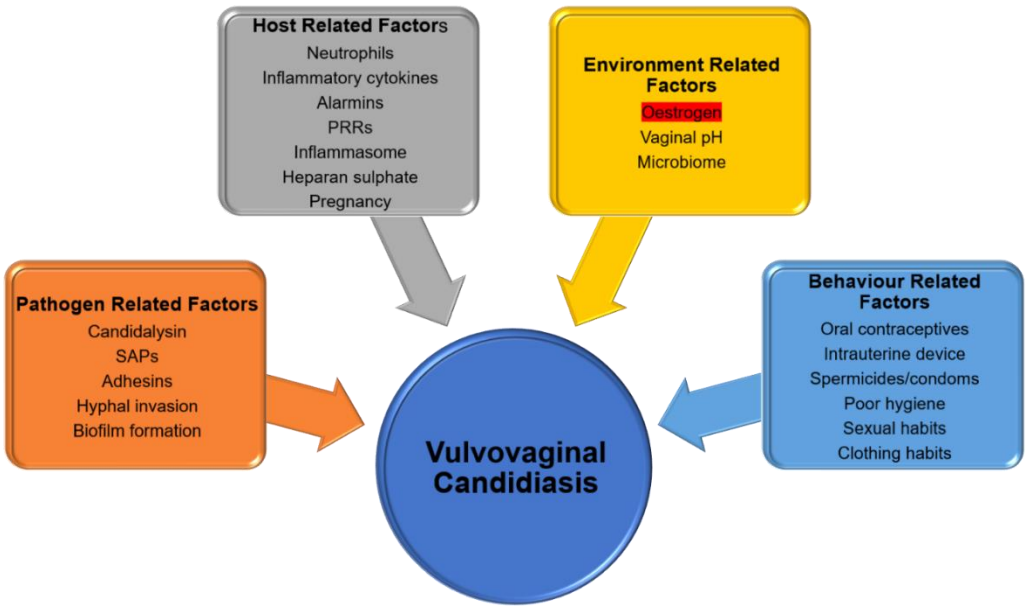

Figure 7.1. Multiple factors contribute to development of VVC. Host, environmental, behavioural and pathogen related factors are necessary to trigger onset of disease and symptomatic infection. Adapted from Willems *et al*., (2020).

Apart from rendering the host vulnerable to VVC, the present study has revealed that adaptation of *C. albicans* to oestrogen promotes fungal immune evasion. The key finding from the current study showed that *C. albicans* adaptation to oestrogen facilitated inhibition of phagocytosis. Reduction in phagocytosis was associated with overexpression of *GPD2* which codes for Gpd2, a moonlighting protein capable of binding FH (a complement regulatory protein) (Luo *et al.,* 2013). The complement system is vital in providing defence against invading pathogens. Nevertheless, microbes have developed strategies to escape the immune effector functions of the complement system. The most common complement evasion strategy employed by microbes is recruiting complement regulatory proteins onto their surface to prevent damage (Luo *et al.,* 2013). Normally complement regulatory proteins control the complement system to avoid damage of host cells and some microbes take advantage of this to recruit the proteins to their surface and survive attack (Meri 2016). Most microbes, including *C. albicans,* bind FH via cell surface proteins and evade complement and its associated immune functions (Zipfel *et al.,* 2013). However, knowledge on whether host niches can promote evasion of complement and its immune effector functions is scarce.

As illustrated in the Figure 7.2, oestrogen drives *C. albicans* innate immune evasion via Gpd2 in multiple ways. The proposed model figure indicates that oestrogen stimulates Bcr1 to trigger transcription of *GPD2*, a gene that encodes Gpd2. Gpd2 translocate to the surface and recruits FH, C3b, C3 and plasminogen to the cell surface.

FH protects the fungal cell from complement attack by preventing formation of C3 convertase, inhibiting activity of C5 convertase, facilitating decay of existing C3 convertase, and promoting Factor I mediated degradation of C3b and C4b (Józsi 2017). Most importantly, cleavage of C3b to iC3b leads to reduced opsonophagocytosis since C3b is one of the major opsonins (Józsi 2017). Like FH, plasminogen, and in particular plasmin cleaves C3b to the inactive form iC3b. The model also suggests that Gpd2 binds to C3 and inhibit its processing into C3b. Collectively, these activities lead to reduced C3b fungal opsonisation and decreased opsonophagocytosis.

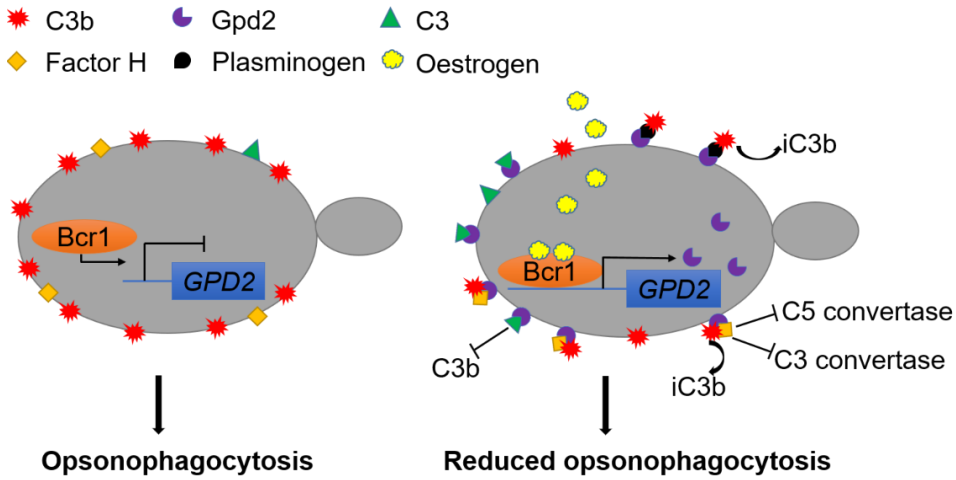

Figure 7.2. Proposed mechanism of oestrogen-induced innate immune evasion. Under standard laboratory conditions C3 and C3b is deposited on the surface of *C. albicans* resulting in effective phagocytosis. However, in the presence of oestrogen, Bcr1 is activated to trigger transcription of GPD2 which codes for Gpd2, a cytosol protein that is later localised on the fungal cell wall. Gpd2 recruits Factor H to the fungal cell surface and facilitate cleavage of C3b, prevents formation of C3 convertase and inhibit activity of C5 convertase. Gpd2 binds C3 to render the complement incapable of being processed to C3b. Plasminogen is also recruited by Gpd2 and degrades C3b to iC3b. Altogether, these activities lead to reduced C3b fungal opsonisation and decreased opsonophagocytosis.

Given the finding on the role of oestrogen *in vitro*, an investigation was carried out to examine whether the hormone could influence virulence of *C. albicans in vivo*. Since zebrafish has a complement system analogous to humans (Zhang *et al.,* 2014), a zebrafish larva disseminated infection model was used to conduct this investigation. Oestrogen promoted virulence of the fungus in zebrafish larvae. This finding adds value to the theory that oestrogen promotes *C. albicans* systemic infections (Relloso *et al.,* 2012). Furthermore, this discovery could explain why VVC is quite prevalent among women of childbearing age even though the complement system is strongly activated in the female reproductive tract in normal circumstances as well during pregnancy and delivery period (Richani *et al.,* 2005, Livson *et al.,* 2020).

7.2 Influence of oral contraceptives on Candidiasis

As previously described, the use of oestrogen based oral contraceptives is a risk factor for VVC (Gonçalves *et al*., 2016) and findings from the present study supports this concept. Surprisingly, women taking oral contraceptives are not prone to systemic candidiasis. A few reasons might be considered as to why this happens. Unlike in the blood stream or host organ tissue, *Candida* already exists in the vagina as a normal flora thus oral contraception/oestrogen simply enhance a shift from asymptomatic to symptomatic infection. Oestrogen enhances fungal overgrowth, epithelial cell adherence and hyphal formation (Dennerstein *et al.,* 2001, Cheng *et al.,* 2006, Hollmer *et al.,* 2006).

Generation of hyphae increases expression of candidalysin which damages the epithelium to stimulate the inflammasome and subsequently trigger recruitment of neutrophils into the vaginal lumen (Moyes *et al.,* 2016, Yano *et al.,* 2018). Via a process called 'neutrophil anergy', the recruited neutrophils are unable to kill *Candida* since heparan sulphate (induced by oestrogen) acts as a competitive ligand for the neutrophil receptor Mac-1 which is vital for fungal recognition and neutrophil driven killing (Yano *et al.,* 2018). Normally, Mac-1 binds to *C. albicans* pH-regulated antigen 1 protein (Pra1p) to trigger antifungal activity (Yano *et al.,* 2018). Oestrogen is also known to promote neutrophil arrest in the vaginal lumen by disrupting the Cxcl1 gradient consequently promoting fungal overgrowth (Lasarte *et al.,* 2015). Therefore, among other factors, the commensal status of *Candida* in the vagina allows oestrogen to effectively promote VVC unlike systemic infections where sites are sterile, and the immune system is prompt to clear the fungus before it causes infection.

Furthermore, the development of VVC is a multifactorial process involving several factors which collectively create a suitable environment for oestrogen to execute its effects (Willems *et al.,* 2020). Some of the risk factors for VVC do not apply in systemic infections. Moreover, the mechanism behind the immunopathogenesis of VVC is unique for mucosal infection and has not been reported in systemic infections.

Finally, although the mechanisms are not yet clear, 17β-estradiol is known to have protective effect against some systemic infections in women. These include Paracoccidioidomycosis and *Coxiella burnetii* Infection (Severo *et al.,* 1998, Leone *et al.,* 2004). Additionally, through activation of the renin-angiotensin-aldosterone system (RAAS), oestrogen protects against a wide range of systemic pathological conditions such as, atherosclerosis, hypertension, vasoconstriction, inflammation, autoimmune diseases, fibrosis, and neurological disorders (Nathan and *et al.,* 1997, Straub 2007, Miller *et al.,* 2008, Gilbert *et al.,* 2014, Yang *et al.,* 2014, Raghava *et al.,* 2017, Moulton 2018). RAAS is a hormone and enzyme driven system that regulates functions of several organs including the heart, kidneys, liver, lungs, vasculature, and pancreas (Nehme *et al.,* 2019). Therefore, it is appealing to suggest that the hormone may have a protective impact against other infectious diseases including systemic candidiasis.

7.3 Effect of oestrogen on microbial virulence

The present study has demonstrated that oestrogen enhanced *C. albicans* virulence. Previous reports also indicate that the oestrogen does promote virulence in other microbes. For example, some *in vitro* studies have demonstrated that oestrogen enhance adherence to mammalian cells by *Chlamydia trachomatis, E. coli*, *S. aureus*, and Group B *streptococci* (Sugarman *et al.,* 1982, Maslow *et al.,* 1988).

In vivo investigations have shown that oestrogen promotes infection by *Listeria monocytogenes*, *Chlamydia psittaci*, Gonococcus, *Salmonella typhimurium* and *Pseudomonas aeruginosa* (Pung *et al.,* 1984, Kita *et al.,* 1985, Barron *et al.,* 1988, Kita *et al.,* 1989, Chotirmall *et al.,* 2012). Therefore, the impact of oestrogen on microbial virulence is a general phenomenon which requires further research as this might involve different mechanisms.

7.4 Moonlighting proteins and fungal virulence

Moonlighting proteins have proved to be vital in promoting microbial virulence (Singh *et al.,* 2020). The current study has described *C. albicans* Gpd2 moonlighting protein as an immune evasion protein, that can be induced by oestrogen to evade complement mediated opsonophagocytosis. Similarly, a previous investigation was carried out to analyse the expression of *Candida* moonlighting proteins under different growth conditions (Karkowska-Kuleta *et al.,* 2019). Growth media with different pH and components was used to mimic possible infection sites like the vagina, oral cavity, gastrointestinal tract, and urinary tract. *C. glabrata*, *C. parapsilosis* and *C. tropicalis* were evaluated and findings showed a diverse expression of moonlighting proteins depending on the growth conditions. Certain growth conditions did not allow expression of some moonlighting proteins while other conditions allowed some proteins to be expressed in abundance.

Some of the moonlighting proteins identified in the study included pyruvate decarboxylase (Pdc11), fructose-bisphosphate aldolase (Fba1), Eno1, Tdh3, phosphoglycerate kinase (Pgk1), transaldolase (Tal1), Adh1 and Gpm1.

Therefore, these findings suggest that apart from oestrogen, other host environments can also induce expression of moonlighting proteins and perhaps promote immune evasion. In addition, results from Karkowska-Kuleta *et al*., (2019) showed that moonlighting proteins in *Candida* species share functional similarities since some proteins exhibited the same functions across the pathogens. This phenomenon was also observed in the present study where the oestrogen induced innate immune evasion facilitated by Gpd2 was observed in *C. albicans* and *C. tropicalis* species.

7.5 Clinical application of the study

Observations from the present study support existing work indicating that oestrogen is a risk factor for VVC. Therefore, to avoid infection, women prone to VVC/RVVC may consider taking alternative contraceptive methods. Similarly, women suffering from menopause symptoms may consider taking alternative therapy to hormone replacement therapy (Sood *et al.,* 2002, Molla *et al.,* 2011).

While data from the present study needs further work, evidence suggest that Gpd2 is an important virulence factor that can be explored as an antifungal drug target.

In addition, knowledge gathered here paves way for investigating whether moonlighting proteins including Gpd2 could be potential diagnostic tools since most of these proteins are intracellular proteins which only become extracellular upon microbial adaptation to host niche.

7.6 Conclusion

Considering the strong link between elevated levels of oestrogen and progression of VVC, the present study unlocks a novel alternative mechanism of how the hormone may influence VVC. Consequently, this improves our understanding on the role of oestrogen in pathogenesis of VVC. Nevertheless, this discovery also unravels various research questions that require further investigations. Some of the areas for further work are highlighted below.

7.6.1 The role of oestrogen binding protein 1 (Ebp1) in oestrogen response

An oestrogen binding receptor known as oestrogen binding protein 1 (Ebp1) has been identified and characterized in *C. albicans* (Powell *et al*., 1984). Ebp1 is similar to old yellow enzyme (OYE) found in *S. cerevisiae*. The two proteins exhibit high homology in their sequence and enzyme activity, however, their binding pattern and reactivity to oestradiol is different (Buckman *et al.,* 1998). Ebp1 is a cytosol protein particularly localised to the vacuoles (Zhao *et al.,* 1995). Ebp1 binds to substances containing phenolic structures (e.g. 17-β-oestradiol) and α, β unsaturated keto/aldehyde structures ((Buckman *et al.,* 1998).

Though with reduced affinity, Ebp1 is also capable of binding to oestrone, dihydrotestosterone, estriol, testosterone and progesterone (Rowland *et al.,* 1992). Through Flavin mononucleotide (FMN) cofactor, Ebp1 facilitates the transfer of electrons from NADPH to α, β unsaturated ketones and aldehydes (Buckman *et al.,* 1998). It is also involved in aromatization and reduction reactions of compounds like 19-nortestosterone (Buckman *et al.,* 1998). Pathologically, an *in vivo* study by Tarry *et al.,* (2005) has demonstrated that interaction of oestradiol with yeast Ebp1 is essential for the colonisation of *C. albicans* in the vagina. In addition, a recent study has also revealed that knockdown of *EBP1* results into inhibition of hyphal formation in *C. albicans* (Kurakado *et al.,* 2017). These studies clearly suggest that *C. albicans* Ebp1 is of medical importance nevertheless the precise role of Ebp1 in *C. albicans* pathogenicity is unknown.

Findings from the present study suggest that Ebp1 may be centrally involved in regulating the activities of Gpd2. Thus, it will be essential to explore the role of Ebp1 in the oestrogen driven immune evasion. This could be accomplished through an *EBP1* gene knockout experiment or by using a reporter gene that could facilitate establishing the pattern and timing of *EBP1* gene expression. This can provide greater insight into understanding the pathogenesis and control of infections caused by *C. albicans* including VVC.

7.6.2 Oestrogen response in other *Candida* species

The current study has shown that oestrogen response varies among *Candida* species (Chapter 3). Among the tested species, *C. albicans* and *C. tropicalis* exhibited the oestrogen mediated innate immune evasion however *C. glabrata, C. dublinensis*, and *C. parapsilosis* did not display the phenotype. A further investigation was carried out on *C. glabrata* to understand why the fungus does not respond to oestrogen like *C. albicans* or *C. tropicalis*. The study was conducted by Hannah Gallagher, an MSc student under my supervision. Here *C. albicans GPD2* gene was cloned into *C. glabrata,* and phagocytosis experiments conducted. Interestingly, a reduction in phagocytosis of oestrogen adapted cell was observed though it was statistically insignificant if compared against the ethanol control (Figure S7.1). This finding further highlighted the importance of Gpd2 in the mechanism behind the oestrogen driven immune evasion.

C. albicans GPD2 is similar to *C. glabrata GPD2* hence it will be vital to investigate why there is variation in response to oestrogen among the two pathogens. *C. albicans* Gpd2 has previously been observed as a cell surface protein when the fungus is treated with normal human serum (Marin *et al.,* 2015). Results from the present study may suggest that treatment of the fungus with oestrogen enhance cell surface recruitment of Gpd2 to perform immune evasion effector functions. Thus, cellular localisation of *C. glabrata* Gpd2 post oestrogen treatment could be done by tagging *GPD2* gene and observe its localisation by fluorescent microscopy.

7.6.3 Signalling pathway analysis

The RNAseq experiment could be utilized to identify signalling pathways involved in facilitating the oestrogen-driven innate immune evasion. Following RNAseq, a gene network and functional analysis of all differentially expressed genes could be performed to identify their associated signalling pathways. Identifying the signalling pathways could be useful in determining ways of inhibiting the currently identified immune evasion strategy.

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9. Appendices

9.1 Appendix A: Supplementary information

Table S4.1. RNAseq data for oestrogen-adapted *C. albicans* cells

Gene name	Fold change	Adjusted p-value	Function
AOX2	4.25	5.04E-11	Alternative oxidase
orf19.3120	3.16	2.36E-07	PDR-subfamily ABC transporter
orf19.355	2.46	0.03	Predicted oxidoreductase activity and role in oxidation-reduction process
orf19.3337	2.18	4.62E-03	Protein of unknown function
GST1	2.17	0.04	Putative glutathione S-transferase
GAP2	-2.02	1.12E-03	General broad specificity amino acid permease
PST1	-2.03	4.12E-08	Flavodoxin-like protein involved in oxidative stress protection and virulence
FOX2	-2.03	6.50E-11	3-hydroxyacyl-CoA epimerase
orf19.94	-2.07	2.09E-06	Protein of unknown function
MLS1	-2.11	8.93E-08	Malate synthase
ICL1	-2.15	1.71E-03	Isocitrate lyase
orf19.7310	-2.15	1.53E-10	Protein with a role in directing meiotic recombination events to homologous chromatids
CAT1	-2.33	4.45E-10	Catalase
ASR1	-2.33	4.69E-11	Heat shock protein
orf19.6688	-2.37	6.53E-03	Protein of unknown function
orf19.2515	-2.4	3.86E-10	ZZ-type zinc finger protein
IHD1	-2.4	1.72E-12	GPI-anchored protein
FDH1	-2.43	3.22E-09	Formate dehydrogenase
HSP30	-2.55	0.04	Putative heat shock protein
orf19.7029	-2.64	7.66E-03	Putative guanine deaminase
HPD1	-2.69	0.03	3-hydroxypropionate dehydrogenase
GIT1	-2.73	1.85E-06	Glycerophosphoinositol permease
HMX1	-2.91	0	Heme oxygenase
FRE7	-3.23	2.25E-03	Copper-regulated cupric reductase
HWP1	-3.72	0.03	Hyphal cell wall protein

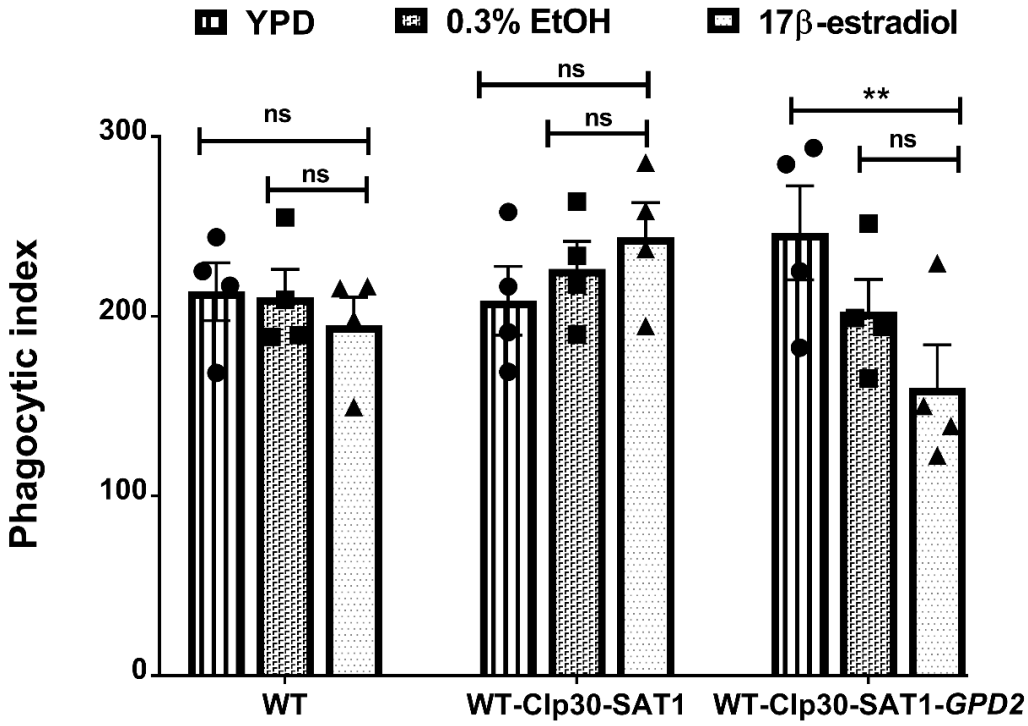

Figure S7.1. Cloning *C. albicans* GPD2 gene into *C. glabrata* promotes innate immune evasion. *C. glabrata* cells (WT), parental control (WT-Cip30-SAT1) and the reconstituted strain (WT-Cip30-SAT1) were grown for 4 h in YPD with or without 10 µM 17β-oestradiol. Cells were harvested, washed in PBS and co-incubated with J774A.1 macrophages for 45 min. Phagocytosis rates were quantified. All data represent the mean ± SEM from at least three independent experiments. Two-way ANOVA, Tukey's multiple comparisons test; **p<0.01.

9.2 Appendix B: Statement of collaborative work and Declaration of authorship

Statement of collaborative work

This is to state that parts of this thesis contain data that were obtained through collaborative work. Collaborators and their contributions are listed below.

1. **Phagocytosis experiment**

In this thesis, Ben Keevan contributed data on phagocytosis experiments of *bcr1*Δ and *rob1*Δ mutants. Ben Keevan was an MRes student at University of Birmingham, UK, doing his project under my supervision.

2. **RNA sequencing**

RNA sequencing was performed at the Environmental Omics Sequencing Facility (University of Birmingham, UK) as a paid service. RNA sequencing data analysis was performed by Dr Fabien Cottier, a research fellow at University of Birmingham, UK.

Declaration of authorship

This is to confirm that Pizga Kumwenda is first author and major contributor to the publication '**Oestrogen promotes innate immune evasion of *Candida albicans* through inactivation of the alternative complement system'** which is currently under revision with Cell reports. However, the paper is available online as a preprint with bioRxiv. Rebecca A. Hall is the corresponding author and Fabien Cottier, Ben Keevan and Hannah Gallagher are co-authors (Appendix I). Text from this publication has been included in the thesis entitled '**Exploring the role of oestrogen in controlling the pathogenicity of *Candida albicans***' submitted to the University of Birmingham on 30th April 2021. Parts of this publication have been included in Chapters 2 to 7.

Signed:

Pizga Kumwenda

Rebecca A. Hall

Dr Rebecca A. Hall

(Corresponding author)

9.3 Appendix C: Publication included in the thesis

This thesis contains data that has been submitted for publication to Cell Reports and is available online as a preprint as indicated below:

1. Kumwenda, P., F. Cottier, B. Keevan, H. Gallagher and R. A. Hall (2020). "Oestrogen promotes innate immune evasion of Candida albicans through inactivation of the alternative complement system." bioRxiv. https://doi.org/10.1101/2020.07.22.207191