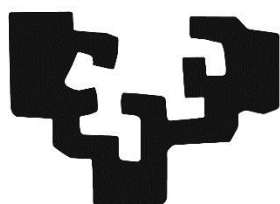


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Universidad
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Euskal Herriko
Unibertsitatea

**Facultad de Medicina y Enfermería
Faculty of Medicine and Nursing**

**Characterization and evaluation of the
antifungal activity of antibodies raised against
Candida albicans germ tube in a rabbit model
of infection and patients with Invasive
Candidiasis**

Departamento de Inmunología, Microbiología y Parasitología
Department of Immunology, Microbiology and Parasitology

Departamento de Enfermería I
Department of Nursing I

Tesis Doctoral – Doctoral Thesis

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Abbreviations and acronyms



α:	anti
A₂₈₀:	absorbance at 280 nanometres
A₄₉₀:	absorbance at 490 nanometres
Ab:	antibodies
aa:	amino acid
Ach1:	acetyl-CoA-hydrolase
Aco1:	aconitase-1
Acs2:	acetyl-CoA-synthetase
ADCC:	antibody-dependent cell-mediated cytotoxicity
Ade17:	5-Aminoimidazole-4-carboxamide ribotide transformylase
Adh1:	alcohol dehydrogenase
AIDS:	acquired immunodeficiency syndrome
Als:	agglutinine like sequence
AM	anti-mycelia
ANOVA:	analysis of variance
Anti-BL:	anti-blastospore antibodies
APC:	antigen-presenting cells
APS/PSA:	ammonium persulfate
ATP:	adenosin triphosphate
Atp2:	F1 β subunit of F1F0 ATPase complex
ATCC:	american Type Culture Collection
β-MN:	β -mannan
BCA:	bicinchoninic acid
BCR:	lymphocyte B receptor
Bel1:	BEL1-like protein 1
Bgl-2:	β 1,3-glycosyltransferase
Big1:	Bad in glucose protein 1
Bmh1:	14-3-3 protein
Bp:	base pair
BSA(BCA):	bovine serum albumine
c-Fos:	transcription factor AP-1
c-Jun	transcription factor AP-1
Car1:	arginase
CCL20:	chemokine ligand 20
cdNA:	complementary deoxyribonucleic acid
CECT:	Colección español de Cultivos Tipo
CAGTA:	anti-germ tube <i>C. albicans</i> antibodies/anticuerpos anti-tubo germinal de <i>C. albicans</i>
CAGTA-enr:	serum fraction enriched of CAGTA
Cdc24:	guanine nucleotide exchange factor Cdc24
CFDA:	5,(6)-carboxyfluorescein diacetate



CFU/UFC:	colony forming unit/unidad formadora de colonias
C_H:	constant region from a heavy chain of an Ig molecule
Chs:	chitin synthases
Cht:	chitinase
CL:	constant region from a light chain of an Ig molecule
Cm:	centimetre
Cpp1:	VH1 family MAPK phosphatase
Csa1:	<i>Candida</i> surface antigen
Csp37:	37 kDa cell surface protein
Cst20:	protein kinase of Ste20p/p65PAK family
CV:	cristal violet
CW-Eno:	cell wall enolase/enolasa de pared
Da:	Dalton
Δ:	deletion
DC:	dendritic cell
DF:	dilution factor
Dfg5:	mannan endo-1,6-alpha-mannosidase DFG5
DiBAC:	bis-(1,3-dibutylbarbituric acid) trimethine oxonol
DMEM:	Dulbecco's Modified Eagle Medium
DMSO:	dimethyl sulfoxide
DNA/ADN:	deoxyribonucleic acid
DTT:	dithiothreitol
Eap1:	enhanced adesions to polystyrene
ECACC:	European Collection of Cell Culture
ECE1:	extent of cell elongation 1
ECL:	enhanced chemiluminiscence
EDTA:	Ethylenediaminetetraacetic acid/ácido etilen diamino tetraacético
EED1:	epithelial escape and dissemination 1
Egd2:	nascent polypeptide-associated complex subunit alpha
EGFR:	epidermal growth factor receptor
Efg1:	enhanced filamentous growth 1
Eft2:	elongation factor 2
Eft3:	elongation factor 1-alpha
ELISA:	enzyme-linked immunosorbent assay
Eno-1:	enolase 1
Erk:	extracellular signal-regulated kinase
Φ:	filter poro size
Fab:	the fragment antigen-binding portion of an antibody
Fas1:	fatty acid synthase
FICT:	fluorescein isothiocyanate-conjugated



Fba1:	fructose-1,6-bisphosphate aldolase
Fbs:	fetal bovine serum
FICT:	fluorescein isothiocyanate/isotiocianato de fluoresceina
FKS:	1,3-beta-glucan synthase
FR:	framework region of an Ig molecule
G-CSF:	granulocyte-colony stimulating factor
Gap1:	glyceraldehyde-3-phosphate-dehydrogenase
Gdp1:	glycerol-3-phosphate dehydrogenase
GlcNac:	<i>N</i> -acetylglucosamine
GM-CSF:	granulocyte-macrophage colony-stimulating factor
GPI:	glycosylphosphatidylinositol
Gpm1:	phosphoglycerate mutase 1
Gpp1:	glycerol-3-phosphatase
Grp:	methylglyoxal reductase
GSC:	beta-1,3-Glucan Synthase Catalytic subunit
GT/TG:	germinal tube
H:	Hour
Hyr1:	glutathione peroxidase-like peroxiredoxin HYR1
HIV:	human immunodeficiency
Hem13:	coproporphyrinogen III oxidase
Her2:	receptor tyrosine-protein kinase erbB-2
Hgt6:	potencial hexose transporter
HRP:	horseradish Peroxidase
HSP:	heat shock protein
Hwp1:	hyphal wall protein
Hxk2:	hexokinase 2
JNK:	c-Jun N-terminal kinase
IC/CI:	invasive candidiasis/candidiasis invasora
ICU /UCI:	intensive care unit /unidad de cuidados intensivo
IFF4:	cell wall protein IFF4
IFI:	indirect immunofluorescent
IFN-γ:	interferon γ
IgG-tot:	immune total serum
Int1p:	integrine
Ipf1786:	Sec18p
Ig:	immunoglobulin
IL:	interleukin
Ilv5:	ketol-acid reductoisomerase
Imh3:	inosine monophosphate dehydrogenase
Ino1:	inositol-1-phosphate-synthase
INT1:	integrin-like protein



Ipp:	pyrophosphatase
IPTG:	isopropyl- β -D-1-thiogalactopyranoside
i.v.:	intravenously
KR:	lysine-arginine
Kb:	kilobase
kDa:	kilodaltons
KEX:	serine protease
Kre:	killer toxin-resistance protein
kV:	kilovolts
Λ :	wave length/longitudinal de onda
L:	litre
LB:	Luria Bertano (broth or agar culture media)
LDH:	lactate dehydrogenase
Leu1:	isopropylmalate isomerase
LDH:	lactate dehydrogenase
Lip:	lipase
Lpd1:	dihydrolipoamide dehydrogenase
LSB:	Laemmli sample buffer
M:	molar
M-CSF:	macrophage colony-stimulating factor
mAb:	monoclonal antibodies
MALT	mucosa-associated lymphoid tissue
Mb:	megabase
Mdh1:	malate Dehydrogenase 1
Met-6:	methionine synthase
MHC:	major histocompatibility complex
mg:	milligram
MTL:	locus mating-Type-Like
MMPs:	matrix metalloproteases
M:	molar
mA:	milliamps
min:	minute
MKP:	MAPK phosphatase
ml:	millilitre
mm:	millimetre
mM:	millimolar
MAPK:	mitogen-activated-protein kinase
MNT1:	alpha-1,2-mannosyl transferase
MOI:	multiplicity of infection
MOPS:	3-(N-Morpholino)propanesulfonic acid
MRPL40:	likely mitochondrial ribosomal protein MRPL40p



MTT:	3-(4,5-Dimethyl-2-thiazolyl)-2,5 diphenyl-2H-tetrazolium bromide
MUC:	mucin
µg:	microgram
µl:	microlitre
µm:	micrometre
µM:	micromolar
NaI:	sodium iodide
NCPF:	National Collection of Pathogenic Fungi
NETs	neutrophils Extracellular Traps
NK-κB:	nuclear factor kappa-light-chain-enhancer of activated B cells
NK:	natural killer
nm:	nanometre
NOT5p/orf6.6343	a protein member of the transcription regulatory CCR4-NOT complex
NPV/VPN:	negative predictive value/valor predictivo negativo
°C:	centigrade degree
OD₆₀₀:	optical density at 600 nanometres
OPC:	oropharyngeal candidiasis
ORF:	open reading frame/marco de lectura abierta
orf6.7858	putative patatin-like phospholipase
PAGE:	polyacrylamide gel electrophoresis
PAMP:	pathogen-associated molecular patterns
PAS:	Periodic acid-Schiff
PBS:	phosphate buffered saline
PBS-BT:	PBS-BSA-Tween20
PBS-SAB:	phosphate buffered saline-Sabouraud broth
PBS-TA:	PBS-Evans blue-Tween20
PCR:	polymerase chain reaction/reacción en cadena de la polimerasa
Pct1:	choline-phosphate cytidylyltransferase
Pdc:	pyruvate decarboxylase
Pdi1:	protein disulfide isomerase
pfu/ufp:	plaque-forming unit/unidades formadoras de placas de lisis
PEG:	polyethylen glycol
PEV7:	Pevion Biotech Target: Recurrent vulvovaginal candidiasis (vaccine)
Pfk1:	phosphofructokinase-1
Pfu:	plaque forming unit
Pga:	predicted Gpi-anchored
Pgk1:	phosphoglycerate Kinase 1
Pgi1:	phosphoglucose isomerase
Phr:	pH responsive



PI3K:	phosphatidylnositide 3-kinase
Pir:	protein with internal repeats
PLB:	phospholipase
PMNs:	polymorphonuclear neutrophils
PMS:	phenazine methosulfate
Pmt:	protein mannosyl transferase
POR1:	mitochondrial outer membrane protein porin 1
POX:	peroxidase
PPV/VPP:	positive predictive value/valor predictivo positivo
Pra1:	PH-regulated antigen
PRR:	pattern recognition receptor
PS:	penicillin-streptomycin
PTH1/PTS1:	proline transporter helper
PVDF:	polyvinylidene fluoride
Qcr2:	subunit 2 of ubiquinol cytochrome-c reductase
Rbf1:	RPG-box binding factor
Rbt:	repressed by TUP1
REA-ICU:	Units of reanimation and intensive care
Rhd:	repressed during hyphae development
Rim101p:	pH-response transcription factor
RIPA:	radio-immunoprecipitation assay buffer
RNA/ARN:	ribonucleic acid
RNA_m/ARN_m:	messenger ribonucleic acid
rmp:	revolutions per minute
RNS:	reactive nitrogen species
ROS:	reactive oxygen species
Rot1:	reversal of TOR2 lethality protein 1
RPMI:	Roswell Park Memorial Institute 1640 Medium
RT:	room temperature (20-27°C)
RTKs:	receptor tyrosin kinases
S:	sensibility/sensibilidad
S/E:	specificity/especificidad
SAB:	Sabouraud
Sah1:	S-adenosyl-L-homocysteine hydrolase
Sap:	secreted aspartyl proteinases
Sc:	Standford DNA Sequencing and Technology Center
ScFv:	single-chain fragment variable
SDA:	Sabouraud dextrose agar
Ssa1:	heat shock protein
Ssb1:	ribosome-associated molecular chaperone
Ssc1:	heat shock protein



Sse1:	heat shock protein homolog
Ssr1:	GPI-dependent protein
SD:	standard deviation
SDS:	sodium dodecyl sulfate
SDS-PAGE:	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM:	scanning electron microscopy
SET1	lysine histone methyltransferase
Shm2:	cytosolic serine hydroxymethyltransferase
Sod:	superoxide-dismutase
Qcr2:	subunit 2 of ubiquinol cytochrome-c reductase
TAE	Tris-Acetate-EDTA buffer
TBS:	tris buffered saline
Tc:	cytotoxic C cell
TCR:	lymphocyte T receptor
TEM:	transmission electron microscopy
TEMED:	tetramethylethylenediamine
TGF-β:	transforming growth factor beta
Th:	type 1 helper cell
Tif1:	ATP-dependent RNA helicase eIF4A
Tkl1:	transketolase
TLR:	toll-like receptor
Tm:	melting temperature
TNF-α:	tumor necrosis factor alpha
Tpi1:	triosephosphate isomerase
Tra98	transposase-like protein
Treg:	regulatory T cells
Tris:	tris(hydroxymethyl)aminomethane
TUP1:	transcriptional corepressor
UPD-glucose:	uridine diphosphate glucose
UPV-EHU:	University of Basque Country
Utr2/Csf4:	cell surface factor
UV:	ultraviolet
V:	volts
V_H:	variable region from a heavy chain of an Ig molecule
V_L:	variable region from a light chain of an Ig molecule
v/v:	volume/volume
vs:	versus
vvc:	vulvovaginal candidiasis
XTT:	2,3-Bis(2-methoxy-4-nitro-sulphenyl)-2H-tetrazolium-5-carboxanilide inner salt
Yhb1:	flavo-hemoglobin



YPD:	yeast extract peptone dextrose
Yel1:	potencial GTP/GDP Exchange factor for ARF
Ywp1:	yeast wall protein 1
WB:	western blotting
w/v:	weight/volume
%:	percent

Summary



In the last decades, the incidence of invasive candidiasis (IC) has dramatically increased. Since *Candida* is a common component of human microbiota, the distinction between invasion and colonization is complicated. Our group of research developed an immunofluorescence assay to detect antibodies reacting specifically against the surface of the germ tubes of *Candida albicans* in those patients with IC. These antibodies appear when an invasive process occurs and have been referred to as CAGTA. The mortality rate of ICU patients was significantly lower in CAGTA-positive patients. Based on this premise, we decided to characterize the antifungal activity of anti-*Candida albicans* germ tube antibodies (CAGTA).

In the first part of this study, we worked with the immune sera obtained from two White New Zealand female rabbits infected intravenously with *C. albicans* blastospores. The sera were fractionated to run the different assays, and were designated as whole serum IgG (total-IgG), antibodies (Abs) against the superficial antigens of *C. albicans* blastospores (anti-BL), CAGTA enriched fraction (CAGTA-enr) and purified CAGTA (CAGTA).

Firstly, we studied the reactivity of total-IgG fraction of two sera raised in the rabbit model of IC using the indirect immunofluorescence technique -serum 16706 anti-mycelium (AM) titer was 1/160 and anti-BL 1/640, while the latest serum 16715 showed an AM titer 1/160 and anti-BL 1/320-. The immunofluorescence assay confirmed that both sera antibodies recognize superficial antigens from blastospores and mycelia of *C. albicans*. In addition, specific IgG titers increased along the infection process, after several *C. albicans* inoculations.

Secondly, we developed several ELISA assays in order to study the kinetics of the antibodies raised in the animal model. We used the following recombinant proteins of *C. albicans*: 14-3-3, Adh1, Als3-N, Eno-1, Hwp1-N, Met-6, and an eluted fraction of approximately 45 kDa of a *C. albicans* cell wall extract (CW-Eno). The kinetics of antibody titers was very similar for the two infected rabbits. The antibody response against Hwp1-N, Eno-1 and CW-Eno augmented along the infection process with a relevant increase after the second inoculation with *C. albicans*. Anti-Als3-N antibodies showed early and moderate titers, while the reaction with Met-6 and Adh1 became significant after the fourth round of inoculation with *C. albicans*. The



highest levels of antibody reactivity were registered for Hwp1 protein. On the contrary, the lowest levels of antibody reactivity were observed for 14-3-3 protein. According to the recorded activity, anti-Adh1 and anti-Met-6 antibodies could contribute to the immunofluorescence reaction against *C. albicans* registered for sera of the rabbit model of IC. Contrarily, the early response of anti-Eno-1, anti-CW-Eno and anti-Hwp1-N suggest that their corresponding proteins play an important role as immunogens during the first stages of *Candida* infection.

In addition to the previously identified antigens recognized by CAGTA, we searched for new targets of these antibodies using a cDNA library prepared in the vector Lambda Zap II from mRNA of *C. albicans* SC5314 growing as mycelia. The screening of the expressed proteins with the CAGTA-enr serum fraction of the IC rabbit model showed 59 positive clones. The analysis of the insert sequence of the positive clones revealed that five of them presented an ORF corresponding to previously described *C. albicans* SC5314 proteins: glucose-6-p-isomerase (PGI1), hyphal wall protein (Hwp1), fatty acid synthase (Fas1), pH-responsive protein 1 (Phr1), and actin (Act1). These proteins are located in different cell compartments and most of them are involved in the formation of hyphae, the cell wall biosynthesis, or are overexpressed during the biofilm formation of *C. albicans*. Antigenicity analyses of these proteins revealed that they contain immunogenic epitopes, although they do not show homologous sequences; consequently, we can affirm that CAGTA gather antibodies with different specificity whose corresponding antigens are related to the mycelial phase of *C. albicans*. The CAGTA-enr fraction from patients with IC also recognized the selected clones and therefore, these antigens could be the basis for the future development of immunization protocols that might protect against *Candida* infections.

In the next section of this work, we measured the effect of CAGTA raised in a rabbit model of IC, on *C. albicans* planktonic cells as well as on the process of biofilm formation and biofilm maturation. The activity was evaluated by colorimetric XTT, Crystal Violet and CFU assays. Viability and cell morphology were assessed by optical, fluorescent or scanning electron microscopy. We observed that *C. albicans* planktonic yeast cells incubated with total-IgG fraction $\geq 50 \mu\text{g/ml}$ from rabbit immune sera showed a drastic reduction of their metabolic activity, as evaluated by



the XTT and CFU assays, and the effect was concentration dependent. The CFU experiment revealed that cell viability was not affected by an irrelevant rabbit IgG (Irr-IgG) when compared to the untreated cells. A parallel experiment with IgG from the CAGTA enriched serum fraction (CAGTA-enr) gave a similar result. However, anti-blastospores antibodies (anti-BL) did not show any significant effect on the metabolic activity of the yeast cells. These data were corroborated with the optical microscopy images of *C. albicans* planktonic cells incubated with increasing concentrations of different IgG fractions.

Total-IgG from rabbit immune serum reduced the growth of cells in a concentration dependent manner. However, CAGTA-enr IgG induced a slight reduction of *C. albicans* growth and, even at the highest concentration (200 µg/ml), short germ tubes could be observed. This paradoxical effect did not correlate with the results of XTT and CFU assays, but electron microscopy images of blastospores and hyphal forms of *C. albicans* treated with CAGTA-enr revealed an altered surface of hyphae. On the contrary, the surface of untreated control cells as well as that of blastospores treated with CAGTA appeared regular and smooth. In addition, fluorescent DiBAC and CFDA staining of *C. albicans* cells treated with CAGTA revealed that these antibodies exerted a fungicidal effect.

The same study was carried out using antibodies from patients with IC. *Candida albicans* yeast cells incubated with total-IgG from immune sera of patients infected by *C. albicans* showed a marked reduction of their metabolic activity, as evaluated by the XTT assay, and the effect was concentration dependent. The effect of serum IgG from patients with high CAGTA titers was greater in comparison with IgG from patients with low CAGTA titers. Anti-blastospores antibodies (anti-BL) did not show any significant effect on the metabolic activity of the yeast cells. The experiment with CAGTA-enr fraction gave similar results to that of total-IgG. When patients were infected with *C. parapsilosis*, *C. glabrata* or *C. tropicalis*, their CAGTA-enr serum fractions reduced *C. albicans* metabolic activity to a different extent, with a mean value of 50-60% inhibition compared to the untreated control.

The optical microscopy images of *C. albicans* planktonic cells incubated with increasing concentrations of CAGTA-enr fractions of human immune sera corroborated the above-mentioned findings. For their part, CAGTA-enr IgG of patients' sera reduced *C. albicans* growth and even at the highest concentration



(200 µg/ml), we still saw short germ tubes in the same way that we had observed with rabbit immune sera.

Electron microscopy images of *C. albicans* treated with CAGTA-enr revealed a wrinkled surface of hyphae with protuberances. On the contrary, the surface of untreated control yeasts and hyphae, as well as that of blastospores treated with CAGTA, appeared regular and smooth. In addition, fluorescent DiBAC and CFDA staining of *C. albicans* cells treated with purified human CAGTA revealed that these antibodies exerted a fungicidal effect.

In conclusion, the activity of anti-blastospore antibodies on the metabolic activity of *C. albicans* did not justify the effect registered for serum total-IgG from a rabbit model of IC or from patients with IC. In addition, the CAGTA-enriched fraction appears as the main responsible for the *in vitro* antifungal effect on viability and metabolic activity of *C. albicans* observed for the IC immune sera.

We also analyzed the phases of biofilm formation of *C. albicans* following different time-based protocols of treatment with CAGTA. Yeast cells of *C. albicans* exposed to CAGTA for the first 24 h of incubation experienced a statistically significant reduction of biofilm production at CAGTA concentrations ≥ 80 µg/ml. When CAGTA were added to a 24 h-old preformed biofilm, according to Protocol II, the maturation of the biofilm was not affected after an additional 24-h incubation period. Similarly, when CAGTA were added after the first 90 minutes of incubation, no significant effect on the development of the 24h-old biofilm was recorded. However, the presence of CAGTA only during the first 90 minutes of incubation, corresponding to the *C. albicans* adhesion step, resulted in a significant reduction of the biofilm biomass -down to 48%- while the metabolic activity remained close to the control group values.

In agreement with the observed effect of CAGTA on *C. albicans* biofilm formation, electron micrographs of 24-hour old biofilms treated with CAGTA exhibited a reduction of the microbial structure density; in addition, fungal filaments appeared rough and with protuberances, when compared to the untreated control.



This is the first report showing that CAGTA reduce *C. albicans* growth, and impair its metabolic activity and ability to form biofilm *in vitro*; this inhibitory activity is focused on the initial phase of biofilm formation.

In the last section of this work, the following experiments performed during a stay at Dr Naglik's laboratory (King's College, London; September-December 2016) aimed to increase our knowledge of *C. albicans* pathogenicity and the ability of Candidalysin peptide to promote damage and generate an immunological response.

Dr Naglik's recent discovery of Candidalysin, the first cytolytic peptide toxin identified in any human fungal pathogen, reveals a novel and unexpected factor by which *C. albicans* induces mucosal pathogenicity and immune activation (Moyes et al., 2016). Candidalysin (SIIGIIMGILGNIPQVIQIIMSIVKAFKGNK; Ece1-III_{62-92K}) is a peptide derived from the Ece1 protein of *C. albicans* SC5314. Ece1p is an *in vitro* substrate for Kex2p, a Golgi-located protease that cleaves proteins after lysine-arginine (KR) motifs. In fungi, it is known that following Kex2p processing, many proteins are subsequently cleaved by Kex1p (also in the Golgi), removing the C-terminal R. To confirm that removal of the C-terminal arginine residue from immature Candidalysin was due to the function of Kex1p, we analyzed the c-Fos binding activity of a *kex1Δ/Δ* null mutant compared to its matched revertant strain. c-Fos translocates to the nucleus and binds to its consensus DNA sequence site, which initiates the induction of downstream pro-inflammatory cytokines. The infection of TR146 epithelial cells with the wild type strain resulted in c-Fos DNA binding activation. While the infection of TR146 cells with *kex1Δ/Δ* did not result in c-Fos DNA binding activation, the exposure to the re-integrant strain *kex1Δ/Δ+KEX1* recovered the activation level induced by the parental strain. This result confirms that Kex1p activity appears as an important requirement for Candidalysin maturation being Candidalysin terminating in K residue the mature peptide.

Furthermore, in order to know if the positively charged C-terminal K residue of Candidalysin might be critical for interacting with negatively charged components of host membranes and promoting damage, we obtained different Candidalysin molecules whose last amino acid K (lysine; positively charged) was substituted with H (histidine; positively charged), E (glutamic acid; negatively charged), or A (alanine; neutral charge). When compared, *C. albicans* Candidalysin variants K, E



and A induced a dose dependent cell damage, estimated as LDH activity of TR146 epithelial cells, while peptide H (positively charged) showed no activity on the epithelial cells. On the other hand, the four *C. albicans* Candidalysin variants induced a strong dose dependent expression of c-Fos and p-MKP1, as well as cytokine secretion (IL-6, IL1 α , IL-1 β , G-CSF, GM-CSF).

Besides, *C. tropicalis*, *C. dubliniensis* and *C. maltosa* Candidalysin analogues induced a strong dose-dependent response in TR146 epithelial cells. The effect of *C. dubliniensis* Candidalysin was stronger than that of *C. tropicalis* and *C. maltosa*. In addition, all molecule variants, even the A ending (neutral charge) peptide, rendered a similar effect to that of the wild peptide (K). All the Candidalysin variants of these three species of *Candida* induced a strong and dose dependent expression of c-Fos and p-MKP1. Moreover, similarly to *C. albicans* Candidalysin, all peptides can induce the secretion of cytokines (IL-1 α , IL-6, G-CSF, GM-CSF and IL-1 β).

Analogue Candidalysin peptides of *C. tropicalis*, *C. dubliniensis* and *C. maltosa* exhibited greater potency *in vitro* than the Candidalysin of *C. albicans*. These variations might be based on the differences in their amino acid structure that would affect their interaction with the epithelium. However, similarly to *C. albicans* Candidalysin, the C-terminal K residue of *C. tropicalis* and *C. dubliniensis* peptides seems not to be critical for interacting with the negatively charged surface of epithelial cells.

Lastly, the peptide K of Candidalysin variant from *C. albicans* 529L unexpectedly produced a paradoxical effect with maximum damage at 10 $\mu\text{g}/\text{ml}$, and a weak dose dependent expression of c-Fos and p-MKP1. Cytokine secretion showed the same unexpected trend registered for the LDH assay.

Generally, we observed that all Candidalysin variants used in our assays presented a dosage effect on epithelial damage induction (LDH assay). In this regard, based on the results obtained, C-terminal K residue seemed not be critical for interacting with the negative charge of epithelial cells surface. These results suggest that despite being different in their amino acid sequences, the function of Candidalysin peptides is conserved.

Index



1	Introduction	1
1.1	Características generales de los hongos	3
1.2	Micosis	4
1.2.1	Micosis superficiales, cutáneas y subcutáneas	4
1.2.1.1	Micosis superficiales y <i>Candida albicans</i>	5
1.2.1.2	Micosis invasoras	6
1.2.1.3	Micosis invasoras provocadas por el hongo <i>Candida</i>	7
1.3	El género <i>Candida</i>	8
1.3.1	<i>Candida albicans</i>	8
1.3.2	Morfología	9
1.3.3	Genoma y ciclo sexual	10
1.4	La pared celular de <i>Candida albicans</i>	11
1.4.1	Polisacáridos	12
1.4.2	Lípidos y material polimérico extracelular	13
1.4.3	Proteínas	13
1.4.3.1	Proteínas unidas a la pared celular mediante enlaces covalentes	13
1.4.3.2	Proteínas solubles	14
1.5	Factores de virulencia	15
1.5.1	Proteínas y su asociación a la morfología y patogenicidad de <i>Candida albicans</i>	16
1.5.1.1	Agglutinin-like sequence 3 (Als3)	16
1.5.1.2	Hyphal regulated 1 (Hyr1)	18
1.5.1.3	Hyphal wall protein (Hwp1)	18
1.5.2	Factores de adhesión	19
1.5.3	Dimorfismo	21
1.5.4	Cambio fenotípico o <i>switching</i>	22
1.5.5	Hidrofobicidad	22
1.5.6	<i>Quorum sensing</i> y tigmotropismo	23
1.5.7	Enzimas hidrolíticos	23
1.5.8	Adquisición de hierro	24
1.5.9	Mimetismo molecular	25
1.5.10	Sinergismo con otros microorganismos	25
1.5.11	Respuesta al estrés	26
1.5.12	Formación de biopelículas (<i>biofilms</i>)	27
1.6	Características antigénicas de <i>Candida</i>	27
1.6.1	Antígenos citoplasmáticos	28
1.6.2	Antígenos de la pared celular	31
1.6.3	Antígenos secretados al medio	31
1.7	Diagnóstico de la CI	32
1.7.1	Diagnóstico microbiológico convencional	32
1.7.2	Diagnóstico microbiológico independiente del cultivo	33
1.7.2.1	Pruebas serológicas	33
1.7.2.1.1	Detección de anticuerpos	33
1.7.2.1.2	Utilización de proteínas recombinantes como biomarcadores	35
1.7.2.1.3	Detección de anticuerpos anti-micelio (CAGTA)	36
1.7.2.1.4	Detección de antígenos	37
1.7.2.2	Detección de componentes no antigénicos	39



1.7.2.2.1	(1,3)- β -D-glucano	39
1.7.2.2.2	Detección de D-arabinitol	40
1.7.2.2.3	ADN	41
1.8	The immune system	42
1.8.1	Innate immunity	42
1.8.2	Adaptive immunity	43
1.8.3	Antifungal immunotherapy	46
1.8.3.1	Enhancement of immune activity	47
1.9	Interaction between <i>Candida albicans</i> and the epithelium and pathogenicity	50
1.9.1	Adhesion	51
1.9.2	Recognition and activation	52
1.9.3	Invasion, induced endocytosis and active penetration	54
1.9.4	Damage	55
1.9.5	Mucosal immunity against <i>Candida albicans</i>	56
1.9.5.1	Ece1p (extent of cell elongation 1)	57
2	Objectives	59
3	Patients, Materials and Methods	65
3.1	Microorganisms and culture media	67
3.1.1	Microorganisms	67
3.1.2	Culture media and growth conditions for <i>Candida</i>	67
3.1.3	Culture media and growth conditions for <i>Escherichia coli</i>	68
3.2	Patients and sera	68
3.3	Sera from with disseminated candidiasis	71
3.4	Production of <i>Candida albicans</i> yeast cells heat-inactivated	72
3.5	Production of <i>Candida albicans</i> mycelia	73
3.6	Serum fractionation, quantification and purification of immunoglobulins (IgG)	73
3.7	Serum titration for CAGTA	75
3.8	Protein assays	77
3.8.1	Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)	77
3.8.2	Coomassie blue staining	79
3.8.3	Membrane Electrotransfer and Ponceau red staining	79
3.8.4	Western blot analysis	80
3.8.5	Determination of protein concentration	81
3.9	Recombinant antigens	81
3.9.1	Recombinant protein induction	82
3.9.2	Protein purification	83
3.10	Detection of antibody in sera by ELISA	83
3.10.1	Checkerboard	85
3.10.2	Standardization of ELISA	85
3.11	Evaluation of CAGTA activity against <i>Candida albicans</i> planktonic cells	86
3.11.1	Determination of <i>Candida albicans</i> metabolic activity: XTT assay	87

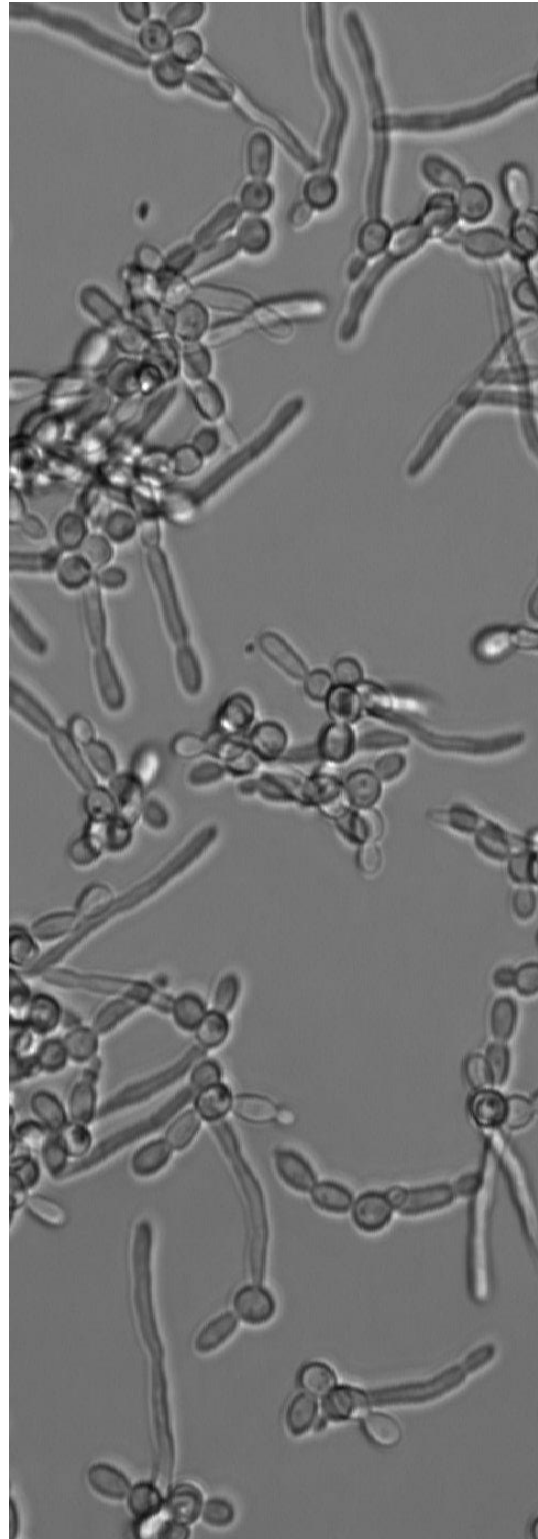


3.11.2	Determination of <i>Candida albicans</i> viability: colony forming units (CFU) assay.....	88
3.12	Evaluation of CAGTA activity against <i>Candida albicans</i> biofilm.....	88
3.13	Microscopy analysis.....	90
3.13.1	Electron microscopy analysis.....	92
3.13.2	Fluorescent analysis.....	92
3.13.3	Optical microscopy analysis.....	92
3.14	Statistical analysis.....	93
3.15	Screening of a <i>Candida albicans</i> cDNA library.....	93
3.15.1	cDNA library amplification.....	93
3.15.2	Titration and ratio of recombinant bacteriophages of the cDNA library.....	95
3.15.3	Library screening using CAGTA.....	98
3.15.4	Polymerase chain reaction and primer design of positive clones.....	101
3.16	Induction of damage by <i>Candida</i> Candidalysins.....	103
3.16.1	<i>Candida albicans</i> strains and growth conditions.....	103
3.16.2	Cell line and growth conditions.....	104
3.17	TransAm assay.....	106
3.18	Epithelial damage and cytokine production determination.....	107
3.18.1	Cell damage assay.....	108
3.18.2	Cytokines determination.....	109
3.19	Protein expression.....	111
3.19.1	Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).....	112
3.19.2	Protein electrotransfer.....	113
3.19.3	Western blot.....	113
4	Results.....	117
4.1	Caracterización de los anticuerpos desarrollados en un modelo animal de candidemia en conejo infectado con <i>Candida albicans</i>.....	119
4.1.1	Reacción de la fracción de IgG totales por inmunofluorescencia indirecta (IFI) frente a <i>Candida albicans</i>	119
4.1.2	Caracterización de los CAGTA mediante ELISA.....	119
4.1.2.1	Producción de Hyphal Wall Protein (Hwp1-N).....	120
4.1.2.2	Evolución de los títulos de los anticuerpos frente a las proteínas recombinantes.....	122
4.2	Selección de antígenos reconocidos por CAGTA mediante rastreo de una genoteca de <i>Candida albicans</i> en el fago λZapII.....	124
4.2.1	Cribado de la genoteca de <i>Candida albicans</i> en λ ZapII.....	124
4.2.2	Localización de regiones inmunogénicas.....	132
4.2.3	Reacción de los CAGTA de pacientes con candidiasis invasora.....	136
4.3	Actividad biológica frente a <i>Candida albicans</i> de los anticuerpos desarrollados en un modelo de candidiasis invasora en conejo.....	137
	Effect of antibodies developed in a rabbit model of candidemia on <i>Candida albicans</i> planktonic cells.....	137
4.3.1	Effect on metabolic activity.....	137
4.3.2	Colony forming unit assay (Effect on viability).....	139
4.3.3	Effect on growth and morphology.....	140



4.4	Actividad biológica frente a <i>Candida albicans</i> de los anticuerpos desarrollados por pacientes con candidiasis invasora	143
	Effect of antibodies developed by patients with invasive candidiasis on <i>Candida albicans</i> planktonic cells	143
4.4.1	Effect on metabolic activity of <i>Candida albicans</i> planktonic cells	143
4.4.2	Effect of CAGTA-enr serum fraction of patients infected with <i>Candida no-albicans</i> species on the metabolic activity of <i>Candida albicans</i>	145
4.4.3	Effect on growth and morphology.....	147
4.5	Effect of CAGTA raised in a rabbit model of invasive candidiasis on <i>Candida albicans</i> biofilm	150
4.5.1	Morphological analysis.....	153
4.6	Effect of Candidalysin of <i>Candida albicans</i> and <i>Candida albicans</i> mutant strains on TR146 epithelial cells line	155
4.6.1	c-Fos activation by wild type and mutant strains of <i>Candida albicans</i>	156
4.6.2	LDH activity to estimate damage induced by <i>Candida albicans</i> Candidalysin variants.....	156
4.6.3	c-Fos and MKP-1 induction by Candidalysin variants of <i>Candida albicans</i>	157
4.6.4	Production of cytokines in oral epithelial cells induced by <i>Candida albicans</i> Candidalysin variants.....	158
4.7	Effect of Candidalysins of <i>Candida dubliniensis</i> and <i>Candida tropicalis</i> on TR146 epithelial cell line	159
4.7.1	LDH activity to estimate damage induced by <i>Candida tropicalis</i> and <i>Candida dubliniensis</i> Candidalysins.....	160
4.7.2	c-Fos and MKP-1 induction by Candidalysins of <i>Candida tropicalis</i> and <i>Candida dubliniensis</i>	161
4.7.3	Production of cytokines in oral epithelial cells induced by <i>Candida dubliniensis</i> and <i>Candida tropicalis</i> Candidalysins.....	162
4.8	Effect of Candidalysin of <i>Candida albicans</i> 529L and <i>Candida maltosa</i> on TR146 epithelial cells line	164
4.8.1	Damage induction of <i>Candida albicans</i> 529L and <i>Candida maltosa</i> Candidalysins	165
4.8.2	c-Fos and MKP-1 induction by Candidalysins of <i>Candida albicans</i> 529L and <i>Candida maltosa</i>	165
4.8.3	Cytokines production in oral epithelial cells induced by <i>Candida albicans</i> 529L and <i>Candida maltosa</i> Candidalysins	166
5	Discussion.....	169
6	Conclusions.....	195
5	References.....	201

1 Introduction





1

1.1 Características generales de los hongos.

El Reino *Fungi* es un vasto y heterogéneo grupo compuesto por organismos eucariotas heterótrofos. De un total entre 1 y 1,5 millones de especies estimadas (Hawksworth, 2001), se han descrito hasta hoy en día aproximadamente 500.000 especies fúngicas (Estrada et al., 2015; Quindós, 2015). Están ampliamente distribuidos en diferentes hábitats, contribuyendo a la degradación de la materia orgánica y participando en los ciclos biológicos.

La mayoría de los hongos establece relaciones simbióticas con otros organismos, dependiendo del tipo de relación éstas pueden dividirse en tres grupos: mutualismo, comensalismo y parasitismo.

El mutualismo es cuando ambos organismos que participan en esta relación se benefician de ella, un claro ejemplo es el mutualismo entre los hongos y las plantas, comúnmente denominadas micorrizas (Bonfante & Anca, 2009). El comensalismo es cuando uno de los dos organismos que participan en la simbiosis es beneficiado mientras que el otro no sufre daño alguno o no trae consigo ninguna ventaja, ejemplo de ello es la colonización por parte del hongo *Candida albicans* de las mucosas (de la cavidad oral, intestinal y vaginal) (Iliev & Underhill, 2013). Se estima que más de la mitad de la población está colonizada por este hongo (Odds, 1988), no suponiendo ningún perjuicio para el individuo en condiciones de equilibrio de la microbiota o con el sistema inmunológico intacto (Naglik, Challacombe, & Hube, 2003). Por otro lado, el parasitismo es cuando en esta relación simbiótica, un organismo (el parásito) se beneficia a expensas de un segundo (el hospedador). Un claro ejemplo de este último tipo de relación se observa en la parasitosis de muchos cultivos de plantas, causando graves daños y pérdidas de cosechas.



Aunque la mayoría de los hongos establecen relaciones simbióticas inocuas para el hospedador, un pequeño número de ellos es capaz de generar importantes enfermedades en humanos, como micotoxicosis, alergias y micosis. Estas últimas incluyen la aspergilosis, candidiasis, coccidiomicosis, cryptococcosis, histoplasmosis, paracoccidiomicosis y rhizomucormicosis, sobre todo en aquellas personas con graves patologías de base.

1.2 Micosis

Las micosis son infecciones provocadas por hongos. Las micosis más comunes son las candidiasis y dermatofitosis, generalmente provocadas por hongos que forman parte de nuestra microbiota natural (Brooks, Carrol, Butel, Morse, & Mietzner, 2011). El género *Candida*, es el hongo más frecuentemente aislado a partir de muestras clínicas procedente tanto de micosis superficiales como invasoras. Por lo general estas se pueden clasificar en tres grandes grupos: micosis superficiales, cutáneas y subcutáneas y micosis invasoras.

1.2.1 Micosis superficiales, cutáneas y subcutáneas

Las micosis superficiales están limitadas a las capas más externas de la piel, cabellos y uñas. El ejemplo más común es la pitiriasis versicolor, causada por *Malassezia*, caracterizada por hipopigmentación o hiperpigmentación de la piel del cuello, hombros, pecho y espalda (Walsh & Dixon, 1996). Las micosis superficiales también pueden afectar a las mucosas orales y genitales con una elevada frecuencia. Es el caso de infecciones producida por *Candida*, *Histoplasma*, *Mucor* y *Trichophyton* (Marques, 2010), generando lesiones inflamatorias pseudomembranosas, eritematosas o hiperplásicas, acompañadas de prurito o aumento de las secreciones en el caso de las vaginitis (Marques, 2010; Quindós, 2015).

Las micosis cutáneas afectan las capas profundas de la epidermis y también incluyen lesiones graves en las extremidades queratinizadas de la piel (uñas y pelos). Estas están producidas por hongos comúnmente denominados dermatofitos, como *Epidermophyton*, *Microsporum* y *Trichophyton* (Cafarchia, Iatta, Latrofa, Gräser, & Otranto, 2013).

Las micosis subcutáneas, por otro lado, están caracterizadas por lesiones en las capas más profundas asociadas a la dermis y epidermis, músculos y fascia. Se



conocen cuatro tipos de micosis subcutáneas: cromoblastomicosis, feohifomicosis, micetoma y sporotricosis (Mitchell, 2010), todas ellas caracterizadas por la implantación traumática del hongo en los tejidos (Quindós, 2015).

1.2.1.1 Micosis superficiales y *Candida albicans*

En el caso de *C. albicans*, las micosis superficiales pueden presentarse con lesiones tanto a nivel cutáneo como a nivel de las mucosas (Quindós, 2015). Generalmente, estas afecciones suelen presentarse en un restringido grupo de personas que presentan enfermedades graves de base, de edades extremas y mayoritariamente en paciente inmunodeficientes (Quindós, 2015; Swidergall & Filler, 2017). Las candidiasis orales, pueden ser agudas o crónicas con lesiones asociadas al hongo *Candida*. Estas afecciones están relacionadas con pacientes con una enfermedad subyacente grave o bien en tratamiento prolongado con antibióticos de amplio espectro, o corticoides inhalados. Pacientes con VIH, diabetes, o con enfermedades autoinmunes presentan un mayor riesgo de sufrir una candidiasis orofaríngea (Swidergall & Filler, 2017). Algunas lesiones están asociadas a neoplasias gástricas (como la candidiasis gástrica) o al uso de una prótesis removible (estomatitis protética). Los pacientes con VIH y neoplasias hematológicas pueden presentar candidiasis pseudomembranosa o esofagitis candidiásica (Quindós, 2015). En las candidiasis orales *C. albicans* es la especie mayoritariamente aislada, seguida por *Candida glabrata* y *Candida tropicalis*. Más raramente se observan aislados clínicos de *Candida parapsilosis*, *Candida krusei*, *Candida dubliniensis* y *Candida guilliermondii* (Revankar & Sobel, 2012).

De gran importancia son también las infecciones de *Candida* en la mucosa vaginal. Este tipo de afección se presenta mayoritariamente en mujeres con diabetes, gestantes o que reciben un tratamiento con antibióticos. Se estima que al menos un 75% de las mujeres han sufrido un episodio de candidiasis vulvovaginal en su vida (Hurley & De Louvois, 1979; Sobel et al., 1998; Uppuluri, Khan, & Edwards, 2017); y que de estas entre el 40-50% sufra una segunda infección (Hurley & De Louvois, 1979). Solo un pequeño porcentaje presenta un cuadro clínico recurrente (Quindós, 2015; Revankar & Sobel, 2012). *C. albicans* es la especie mayoritariamente aislada (90%) a partir de muestras recogidas de mucosa del tracto vaginal, seguida por



C. glabrata, *C. tropicalis*, *C. parapsilosis* y *C. krusei* (Gonçalves et al., 2016; Moran, Coleman, & Sullivan, 2012).

1.2.1.2 Micosis invasoras

Las micosis invasoras están causadas por hongos patógenos primarios y oportunistas. Generalmente los primeros son capaces de generar una infección en individuos sanos y utilizan el tracto respiratorio como principal vía de acceso. En este grupo se incluyen patógenos como *Coccidioides immitis*, *Histoplasma capsulatum*, *Blastomyces dermatitidis*, y *Paracoccidioides brasiliensis*. Estos hongos infectan a la mayoría de las personas con quienes entran en contacto, aunque es muy bajo el porcentaje de las que enferman y desarrollan una enfermedad grave (Quindós, 2015). Por otro lado, los hongos oportunistas son capaces de desarrollar una micosis invasora en aquellos individuos ya previamente comprometidos como los que padecen neoplasias, trasplantados, paciente de cirugía o los que padecen una infección por el virus de VIH. Las principales vías de acceso suelen ser el trato respiratorio, el aparato digestivo, dispositivos como catéteres intravenosos y la propia microbiota (Walsh & Dixon, 1996). Las micosis invasoras están provocadas mayoritariamente por el hongo *Candida* (Enoch, Yang, Aliyu, & Micallef, 2017), aunque hay muchos casos de aspergilosis en aquellos pacientes receptores de un trasplante de progenitores hematopoyéticos con tratamiento antifúngico sin actividad contra *Aspergillus* (Quindós, 2015). Se incluyen en este grupo también *Cryptococcus neoformans*, *Penicillium marneffeii*, Zygomycetos, *Trichosporon beigeli*, y *Fusarium* spp.

Las tasas de mortalidad de las micosis invasoras son muy elevadas, cercana al 30% en el caso de las candidiasis (Gudlaugsson et al., 2003; Quindós, 2015); más del 50% en el caso de las aspergilosis y 90-100% en mucormicosis o escedosporiasis (Quindós, 2015). Anualmente, por cada millón de habitantes, se estiman entre 20 y 200 casos de candidiasis invasoras (CI), 20-60 de criptococcosis y 10-30 de aspergilosis (Quindós, 2015).



1.2.1.3 Micosis invasoras provocadas por el hongo *Candida*

Aunque las infecciones de las mucosas provocadas por el hongo *Candida* son lesiones molestas que pueden llegar a disminuir la calidad de vida de la persona directamente interesada, el problema pasa a ser mucho más grave cuando *Candida* consigue cruzar la barrera epitelial y entrar en el torrente sanguíneo generando infecciones invasoras que llegan a amenazar la vida del paciente.

El desarrollo de una CI va a depender de dos aspectos fundamentales, en primer lugar los factores de virulencia de la especie de *Candida* que genera la infección, y por otro lado la situación clínica de un paciente (Quindós, 2015).

La especie mayoritariamente aislada es *C. albicans*, causando entre el 50 y 60 % de todas las candidiasis. En total más del 90% de los casos de CI es atribuible a *C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis* y *C. krusei* (Moran et al., 2012; Perlroth, Choi, & Spellberg, 2007; Pfaller & Diekema, 2007). Menos frecuente son *C. dubliniensis*, *C. guilliermondii*, *Candida lusitaniae*, *Candida norvegensis* y *Candida rugosa* (Quindós, 2015).

De particular importancia y preocupación es la infección fúngica invasora provocada por el patógeno emergente *Candida auris*. Esta última, es una especie multirresistente descrita por primera vez en el 2009, aislada a partir de una secreción ótica de un paciente japonés (Sato et al., 2009; Uppuluri et al., 2017). Presenta una elevada tasa de mortalidad, y sus infecciones son frecuentes en pacientes hospitalizados con estancias prolongadas, causando infecciones en sangre, en heridas y de oídos (Lee et al., 2011).

A pesar de la introducción de nuevos antifúngicos, la tasa de mortalidad relacionadas con el hongo *Candida* sigue siendo muy elevada (cercana al 30%) como ya se ha comentado anteriormente; además se estima una incidencia de candidiasis de 4,9 casos por cada 100.000 habitantes (Almirante et al., 2005; Pfaller & Diekema, 2007).

A la hora de prevenir la CI existen hoy en día cuatro tipos de estrategias. La primera está basada en la reducción del uso de dispositivos como los catéteres, o bien en la reducción de su tiempo de aplicación, acompañado por una concienciación del



personal sanitario a la hora de su correcta y estéril aplicación. La segunda está basada en la administración de una terapia antifúngica en pacientes de alto riesgo. Esta estrategia se está mostrando de gran utilidad en pacientes trasplantados o que sufren una neutropenia prolongada. La tercera estrategia está basada en una inmunización pasiva o en vías alternativas que incrementen la respuesta inmunológica del individuo. La cuarta y última estrategia está basada en una inmunización activa con la directa aplicación de una vacuna (Spellberg, Marr A. Kieren, & Filler, 2012).

1.3 El género *Candida*

El género *Candida* fue descrito por primera vez por Berkhout y colaboradores en 1923 (Berkhout, 1923), pertenece al Phylum *Ascomycota*, Clase *Hemiascomycetes*, dentro del Orden *Saccharomycetales*. Incluye alrededor de 150 especies, y la mayoría crecen como hongos levaduriformes unicelulares. Sólo una pequeña fracción puede adquirir otras morfologías como las de pseudohifas (*Candida tropicalis*) o hifas (*Candida albicans*, *Candida dubliniensis*) (Moran et al., 2012).

De este amplio grupo solo 15 especies han resultado ser patógenos humanos: *C. albicans*, *C. parapsilosis*, *C. tropicalis*, *C. glabrata*, *C. krusei*, *C. guilliermondii*, *Candida lusitaniae*, *C. dubliniensis*, *Candida pelliculosa*, *Candida kefyr*, *Candida lipolytica*, *Candida famata*, *Candida inconspicua*, *Candida rugosa* y *C. norvegensis* (Pfaller & Diekema, 2007; Quindós, 2015; Uppuluri et al., 2017). A este grupo hay que añadir la especie *C. auris* (Uppuluri et al., 2017).

1.3.1 *Candida albicans*

C. albicans es un hongo dimórfico que pertenece a la familia *Candidaceae* (Kurtzman, Fell, & Boekhout, 2011). Habitualmente forma parte de la microbiota normal de las mucosas de la cavidad oral, del tracto gastrointestinal y urogenital de los seres humanos. Sin embargo, es un patógeno oportunista y en determinadas condiciones puede desencadenar una infección, que va desde una candidiasis superficial hasta invasora (CI). Estas infecciones no se desarrollan únicamente en personas con graves enfermedades de base, sino también en individuos sanos que presentan leves alteraciones de la propia microbiota como resultado de un tratamiento antibiótico



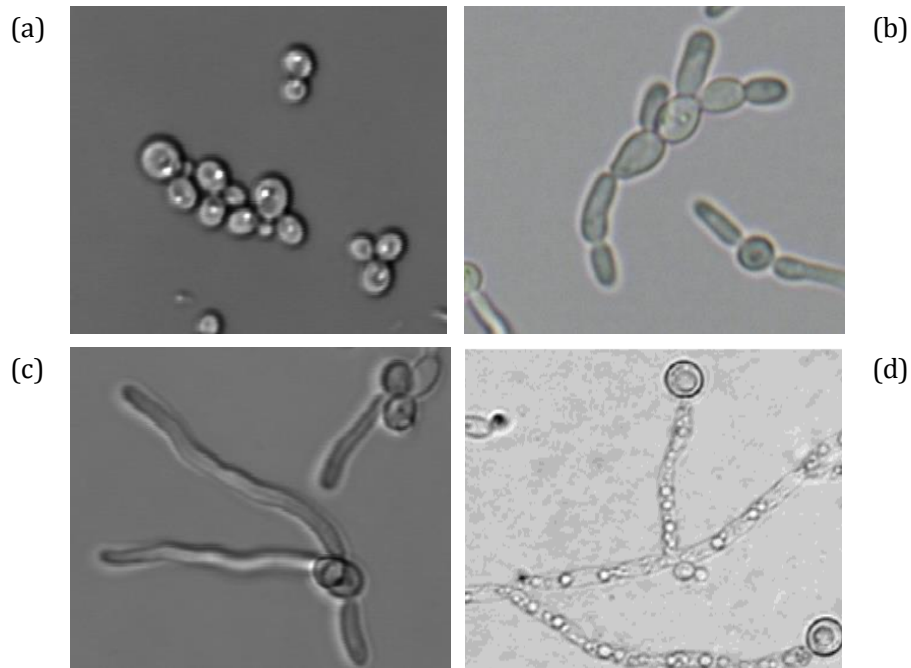
o infecciones vaginales debidas a cambios hormonales en mujeres saludables (Moran et al., 2012).

C. albicans es un organismo heterótrofo. Aunque puede crecer en un medio de cultivo definido con una fuente de carbono (glucosa), nitrógeno (sales de amonio, no nitratos) y fosfato, necesita algunos factores como la biotina, tiamina y otros componentes del grupo vitamínico B. Mediante la fermentación asimila azúcares como la glucosa y la maltosa, pero no lactosa o sacarosa. Puede asimilar también una gran variedad de carbohidratos, lactato y acetato, pero no utiliza la lactosa, celobiosa, rafinosa, inositol y D-ribosa (Odds, 1988). Es capaz de crecer en anaerobiosis en un rango de temperaturas entre 20 y 45°C.

1.3.2 Morfología

Dependiendo de las condiciones ambientales, *C. albicans* puede presentarse bajo distintas morfologías: blastospora, pseudohifa, hifa o clamidospora (Gale & Berman, 2012; Whiteway & Bachewich, 2007) (Figura/Figure 1.1). Esta habilidad de cambiar de morfotipo es un aspecto importante para su virulencia (Sudbery, Gow, & Berman, 2004).

Por lo general, en el laboratorio, los cultivos de *C. albicans* a bajas temperaturas (inferiores a 33°C) y pH ácido contienen mayoritariamente blastosporas (Berman, 2006), células ovoides entre 4-6 µm de diámetro, que se reproducen por gemación. Mientras que a temperaturas de 37°C y pH neutro, estas células levaduriformes se transforman en filamentos (micelios), sobre todo en respuesta a estímulos externos como la presencia de suero, N-acetilglucosamina y prolina. La formación de pseudohifas está caracterizada por la presencia de blastosporas que permanecen unidas después de su gemación; este proceso se observa con valores intermedios de temperatura y pH (Berman, 2006). Por último, las clamidiosporas son células grandes redondeadas de 6-20 µm de diámetro que se pueden observar en los ápices de las hifas. Presentan una pared muy gruesa y son una forma de resistencia. Poseen una actividad metabólica muy baja y pueden originar una nueva célula o degenerar (Quindós, 2015; Staib & Morschhäuser, 2007).



Figura/Figure 1.1 Se representan las cuatro diferentes morfologías de *C. albicans*. Blasposporas (a), pseudohifas (b), hifas (c) y clamidosporas (d, Staib & Morschhäuser, 2007).

La mayoría de las especies de *Candida* puede adquirir una morfología levaduriforme o de pseudohifas, mientras que el morfotipo filamentosamente únicamente se encuentra en *C. albicans* y *C. dubliniensis*.

1.3.3 Genoma y ciclo sexual

C. albicans presenta un genoma diploide, cuya secuencia completa fue publicada por el Centro de Genómica de Stanford (Stanford Genome Center, EEUU), a partir de la cepa de referencia SC5314 (Jones et al., 2004). Su genoma, cuyo tamaño oscila entre 15 y 16 Mb, está compuesto por ocho pares de cromosomas, siendo el cromosoma 1 el más grande y el 7 el más pequeño. El octavo cromosoma contiene el ADN ribosómico, por lo que se denomina R, y su tamaño varía de 3,2 a 4 Mb. Una de las características más interesantes del genoma de *C. albicans* es la aparición de reordenamientos cromosómicos numéricos y estructurales para generar diversidad genética, denominados polimorfismos de longitud cromosómica (contracción/expansión de repeticiones), translocaciones recíprocas, deleciones cromosómicas y trisomías. Estas alteraciones cariotípicas conducen a cambios en el fenotipo como estrategias de adaptación (Larriba & Calderone, 2008; Magee, 2007).

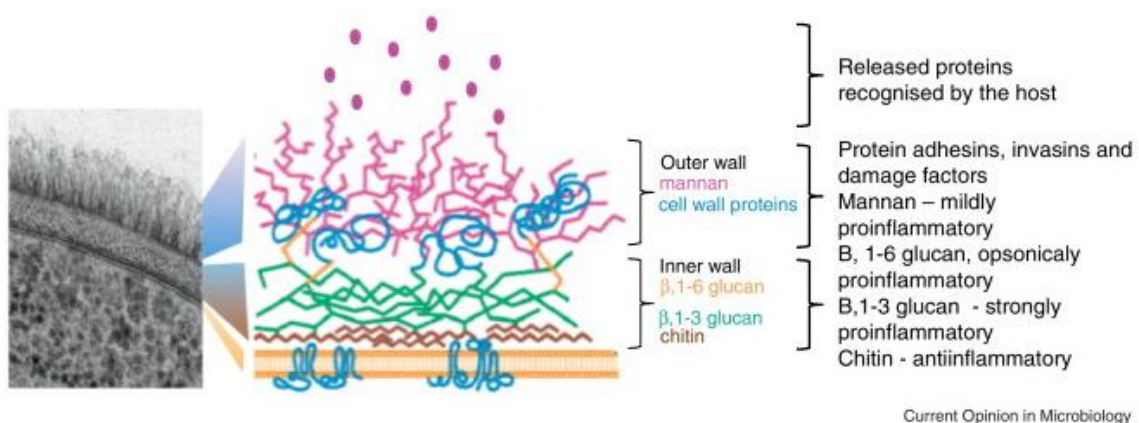


Durante mucho tiempo se consideraba *C. albicans* como un organismo diploide asexual, sin embargo, la secuenciación de su genoma, en 1999 por Hull y Johnson, llevó al descubrimiento del locus MTL (mating-type locus), localizado en el cromosoma 5 (Hull & Johnson, 1999). Siendo *C. albicans* un organismo diploide, presenta dos copias del locus MTL, cuya mayoría son heterocigotos (MTLa/ α). El locus MTLa contiene los genes *MTLa1* y *MTLa2* y de la misma forma el locus MTL α contiene los genes *MTL α 1* y *MTL α 2*. El cambio fenotípico o switching de las células de blanco a opaco es controlado por el locus MTL y es un paso crítico en el ciclo sexual de *C. albicans*, siendo el fenotipo opaco (homocigoto para alguna de las dos presentaciones MLT) la forma competente para llevar a cabo el apareamiento (Soll, 2014).

1.4 La pared celular de *Candida albicans*

La pared celular de los hongos les confiere protección. Es un órgano dinámico que puede reestructurarse gracias a diferentes mecanismos en respuesta a señales internas y externas. Está en contacto directo con el exterior de la célula y en el caso de los hongos patógenos, como *C. albicans*, interactúa con el huésped así como con la microbiota (Munro & Richard, 2012).

La pared celular está estructurada en capas (Figura/Figure 1.2). Mayoritariamente está constituida por polisacáridos (80-90%), y entre 5-10 % de proteínas, mientras que lípidos y sales inorgánicas ocupan solo una pequeña parte.



Figura/Figure 1.2 Esquema de la pared celular de *C. albicans* (Gow & Hube, 2012).



1.4.1 Polisacáridos

Los polisacáridos de la pared celular de *C. albicans* son el β -glucano, quitina y manano.

Los β -glucanos son polímeros de D-glucosa unidos mediante enlaces β -glicosídicos que junto a la quitina constituyen el componente estructural de la pared celular. Este polisacárido unido a la quitina se encarga del mantenimiento de la pared y de su rigidez estructural.

El $\beta(1-3)$ -glucano es un polímero de residuos de glucosa sintetizados a partir de UPD-glucosa (uridina difosfato glucosa) por una $\beta(1-3)$ glucano sintasa. Esta enzima está formada por una subunidad catalítica (Fks/Gsc) y una subunidad reguladora (Rho1). En los hongos en general, existe más de una subunidad catalítica con funciones diferentes. En el caso de *C. albicans*, la subunidad Fks1/Gsc1 es la que mayoritariamente actúa en la síntesis del $\beta(1-3)$ glucano y es diana de algunos antifúngicos como las equinocandinas (Douglas et al., 1997; Munro & Richard, 2012). Además es un componente muy importante asociado al reconocimiento de patrones moleculares y punto de unión con el receptor dectina-1, relacionado con la respuesta inmunológica innata (Munro & Richard, 2012).

En el caso de $\beta(1-6)$ -glucano, su síntesis no está totalmente aclarada, aunque en *Saccharomyces cerevisiae* los genes *BIG1*, *KRE1*, *KRE6*, *KRE9*, *KRE11* y *ROT1* se han visto relacionados con su síntesis (Shahinian & Bussey, 2000).

La quitina es un homopolímero lineal formado por residuos de N-acetilglucosamina mediante enlaces $\beta(1,4)$ -O-glicosídicos. Se une por enlaces β -1,6 covalentes al β -glucano. Su síntesis se debe a una familia de enzimas localizadas en la membrana celular. Los mejores caracterizados en *C. albicans* son Chs1, Chs2, Chs3 y Chs8 (Munro & Gow, 2001). Aunque la quitina representa solo una pequeña fracción de la pared celular del hongo (0,6-2,7%), en la fase micelial aparece cinco veces más concentrada (Chiew, Shepherd, & Sullivan, 1980). Su rol en la respuesta inmune todavía permanece desconocido, pero si se ha reconocido su papel como molécula inmunomoduladora (Lenardon, Munro, & Gow, 2010).



Finalmente, los mananos son polímeros de D-manopinarosa unidos mediante enlaces α -glicosídicos. Este componente a su vez se une covalentemente a residuos de serina o treonina de proteínas mediante enlaces O-glicosídicos, formando las manoproteínas. Representan el componente principal de la matriz de la pared celular de *C. albicans* en el que se encuentran embebidos los polímeros estructurales quitina y β -glucano. Los mananos confieren hidrofobicidad a la pared celular (Ruiz-Herrera, Victoria Elorza, Valentín, & Sentandreu, 2005).

1.4.2 Lípidos y material polimérico extracelular

Los lípidos son el componente minoritario de la pared celular de *C. albicans*. Entre ellos se incluyen los fosfolípidos, esteroides, triglicéridos y ácidos grasos libres. Su proporción varía dependiendo de la morfología y la edad de la célula (Ghannoum, Burns, Elteen, & Radwan, 1986; Gil-Navarro et al., 1997). Los lípidos se han visto involucrados en la adhesión, protección y señalización celular de *C. albicans* (Mille et al., 2004).

Por otro lado el material polimérico extracelular está compuesto por carbohidratos (65-82%), proteínas (7%), fósforo (0,5%) y glucosaminas (1,5%). Este material juega un papel en la adhesión de la levadura y en la estabilidad y desarrollo de biopelículas (Reese & Guggenheim, 2007).

1.4.3 Proteínas

Las proteínas representan alrededor del 35% de la pared celular de *C. albicans*, y juegan un papel importante (Munro & Richard, 2012). Se pueden dividir en tres grandes grupos; el primero está formado por proteínas unidas covalentemente mediante un anclaje GPI (glicofosfatidilinositol) al β -glucano; el segundo grupo está formado por las proteínas Pir (proteínas con repeticiones internas) unidas al β 1,3-glucano mediante enlaces covalentes sensibles al tratamiento alcalino; y el último incluye proteínas solubles no unidas a la pared mediante enlaces covalentes.

1.4.3.1 Proteínas unidas a la pared celular mediante enlaces covalentes

Estas proteínas juegan un papel muy importante en el mantenimiento de la forma celular así como de la virulencia de *C. albicans* (Klis, Sosinska, de Groot, & Brul,



2009). *C. albicans* ha desarrollado numerosos mecanismos para poder sobrevivir en el huésped, diseminarse e invadir los tejidos. En la mayoría de estos mecanismos se ven implicadas estas proteínas, atribuyéndoles diferentes aspectos de virulencia. Algunos de estos aspectos son la adhesión y formación de biopelículas en los que están implicadas la secuencia parecida a aglutininas (Als3), proteína de la pared micelial (Hwp1), antígeno de superficie de *Candida* (Csa1), predictor de anclaje GPI (Pga10), proteína reprimida da TUP1-corepressor transcripcional (Rbt5) y proteína de adherencia aumentada a poliestireno (Eap1) (Bastidas, Heitman, & Cardenas, 2009; Garcia-Sanchez et al., 2004; Klotz et al., 2007; Nobile et al., 2008b; Nobile, Nett, Andes, & Mitchell, 2006; Perez et al., 2006). También se ha visto su implicación en la hidrofobicidad y adhesión de la pared celular (Eap1) (Li & Palecek, 2003), así como en la adquisición de hierro (Als3 y Rbt) (Munro & Richard, 2012).

Las proteínas unidas covalentemente mediante puntos de anclaje GPI, forman un complejo altamente conservado caracterizado por un grupo lipídico con un *myo*-inositol, una N-acetilglucosamina, tres moléculas de manosa y un grupo fosfoetanolamina que se une a las proteínas mediante un enlace amida (Munro & Richard, 2012). Basándonos en estudios proteómicos se han identificado al menos 31 proteínas GPI: Pga4, Pga10, Pga30, Pga31, Pga45, Pga59, Pga62, Als1, Als2, Als3, Als4, chitinasa (Cht2), Cht11, Csa1, manano endo-1,6- α -manosidasa DFG5 (Dfg5), Eap1, Ecm33, Hwp1, proteína sensible al pH (Phr1), Phr2, proteína con repeticiones internas (Pir1), proteína reprimida durante la formación de las hifas (Rhd3), Rbt1, Rbt5, proteasa aspártica secretada (Sap9), Sap10, superóxido dismutasa (Sod4), Sod5/Pga3, proteína unida covalentemente a la pared celular (Ssr1), glucosidasa extracelular (Utr2/Csf4) y proteína de la pared de blastosporas (Ywp1) (Castillo et al., 2008; Chaffin, 2008; de Groot, Hellingwerf, & Klis, 2003; Klis et al., 2009; Maddi, Bowman, & Free, 2009; Sosinska et al., 2008).

1.4.3.2 Proteínas solubles

Las proteínas solubles se caracterizan por mantener uniones débiles a la pared celular y pueden ser extraídas mediante la utilización del detergente iónico SDS (dodecilsulfato sódico) o mediante agentes reductores como el β -mercaptoetanol o el ditiotreitól (DTT). Algunas de estas proteínas presentan funciones enzimáticas



relacionadas con la estructura de la pared celular, o bien asociadas a la glicolisis, estrés, fermentación, o bien con funciones inmunomoduladoras (Munro & Richard, 2012).

Este amplio grupo de proteínas a su vez se puede dividir en dos subgrupos. El primer grupo está caracterizado por aquellas proteínas que no pierden el péptido señal, como la antígeno regulador de pH (Pra1) y la $\beta(1,3)$ -glicosiltransferasa (Bgl2) (Marcil et al., 2008; Sarthy et al., 1997). El segundo está caracterizado por aquellas que pierden el péptido señal y pueden ser exportadas por rutas no clásicas. En este grupo se incluyen la enolasa (Eno-1), la alcohol deshidrogenasa (Adh1), piruvato quinasa, fosfoglicerato mutasa, y metionina sintasa (Met-6) (Cleves, Cooper, Barondes, & Kelly, 1996).

Algunas de estas proteínas, y concretamente la Eno-1 y la Adh1, están clasificadas como citoplasmáticas, pero se ha observado su presencia también en la pared celular de *C. albicans*. Esta doble localización hace que algunos autores las definan como multifuncionales (moonlight), otros las consideran el resultado de una contaminación con componentes citoplasmáticos a lo largo del proceso de extracción (Klis, de Jong, Brul, & de Groot, 2007), mientras que otros autores consideran esta segunda localización como genuina (Nombela, Gil, & Chaffin, 2006). En este sentido se ha visto como el aminoácido 169 de la extremidad N-terminal de la Eno-1 es suficiente para dirigir la proteína a la superficie celular, apoyando por lo tanto el argumento de su doble localización, tanto en el citoplasma como en la pared celular (López-Villar et al., 2006).

1.5 Factores de virulencia

Hasta hace poco, era creencia común que el hongo *Candida* actuaba de forma pasiva en el proceso de infección, pero hoy en día se sabe que el hongo actúa activamente utilizando unos mecanismos llamados factores de virulencia (Gonçalves et al., 2016). Estos elementos sirven al hongo para poder reconocer y establecer un contacto con el organismo hospedador y junto con la capacidad de secretar enzimas se incrementa su grado de virulencia (Calderone & Fonzi, 2001). Entre ellos se incluyen, factores de adhesión, de formación de biopelículas, producción y secreción de enzimas hidrolíticos, formación de hifas y “switching” fenotípico.



1.5.1 Proteínas y su asociación a la morfología y patogenicidad de *Candida albicans*

La morfología de *C. albicans* varía entre blastospora, pseudohifa e hifa, y por tanto un número importante de cambios deben de ocurrir en la pared celular del hongo. Existe una gran variedad de señales que favorece *in vitro* la formación de los micelios tales como el aumento de la temperatura (37°C), pH neutro o la adición de suero o N-acetilglucosamina, produciendo además de un aumento en la síntesis de la quitina (Munro, Schofield, Gooday, & Gow, 1998), la sobreexpresión de determinadas proteínas a lo largo de la formación de los tubos germinales, tales como, la Hwp1 (Staab, Bradway, Fidel, & Sundstrom, 1999), proteína regulada por las hifas (Hyr1) (Bailey, Feldmann, Bovey, Gow, & Brown, 1996), Als3/Als8 (Hoyer, Payne, Bell, Myers, & Scherer, 1998), Rbt1 (Braun, Head, Wang, & Johnson, 2000), Csa1/Wap1 (Braun et al., 2000) y una proteína inducida durante la formación de hifas (Ihd1/Pga36) (Nantel et al., 2002), mientras que la proteína Ywp1 está asociada al crecimiento levaduriforme (Granger, Flenniken, Davis, Mitchell, & Cutler, 2005). En este sentido, puesto que el crecimiento de *C. albicans* está directamente relacionado con la capacidad del hongo para invadir los tejidos del huésped, el estudio comparativo del proteoma de la pared es una herramienta útil para la búsqueda de nuevos componentes antigénicos específicos de la fase micelial tanto para diagnóstico como tratamiento de la CI.

La totalidad de estos cambios que ocurren en la superficie celular, tanto de las blastosporas como de los micelios, así como su antigenicidad y la exposición de componentes fúngicos a las células del sistema inmune del huésped van generando diferentes respuestas inmunológicas (Munro & Richard, 2012).

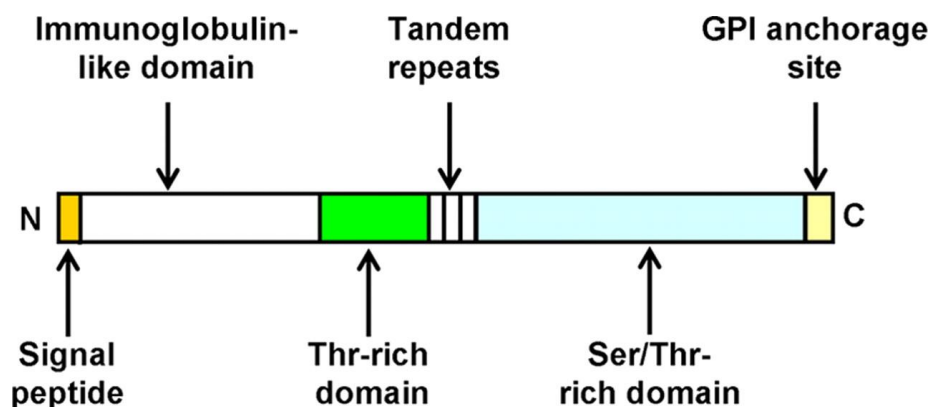
1.5.1.1 Agglutinin-like sequence 3 (Als3)

La proteína Als3 está implicada al igual que otros miembros de la misma familia en la adhesión e interacción con las células del huésped (Zordan & Cormack, 2012). Células de *C. albicans* que han perdido el gen que codifica para esta proteína presentan una reducción en la adhesión y daño a células epiteliales y endoteliales (Phan et al., 2007; Zhao et al., 2004).



Esta proteína comparte la misma estructura que las otras ALS de la misma familia. Esta familia de proteínas presenta un péptido señal en el extremo N-terminal seguido por un dominio de 300 aminoácidos (aa) y un dominio de 401 aminoácidos rico en treoninas con estructura de láminas β (Gaur & Klotz, 1997; Hoyer & Hecht, 2001; Phan et al., 2007; Sheppard et al., 2004) (Figura/Figure 1.3).

Muchas de las proteínas Als presentan función de adhesión, y el punto de unión para la mayoría de los sustratos se localiza en el extremo N-terminal (Rauceo et al., 2006; Sheppard et al., 2004). La Als3 presenta un dominio central caracterizado por un número variable de repeticiones en tándem 36 aa (Sheppard et al., 2004). Estas repeticiones están ricas en serina y treonina, están expuestas en la superficie celular y son necesarias para el proceso de adhesión (Loza et al., 2004; Rauceo et al., 2006). La extremidad carboxi-terminal (C-terminal) de las proteínas Als es rica en serina y treonina y está altamente glicosilada. Contiene sitios de unión al GPI, lo que las une covalentemente a la pared celular (Gaur & Klotz, 1997; Kapteyn et al., 2000).



Figura/Figure 1.3 Esquema de la estructura de la proteína Als3 (Liu & Filler, 2011).

Además de estar implicada en el proceso de adherencia e invasión, la Als3 se ha relacionado con la adquisición de hierro (Zordan & Cormack, 2012).

De la misma forma, la extremidad N-terminal de esta proteína se está estudiando con fines terapéuticos. Actualmente la vacuna frente a las candidiasis vulvovaginales basada en el extremo N-terminal de la Als3 se encuentra en la fase clínica 1b/2a (Uppuluri et al., 2017).



1.5.1.2 Hyphal regulated 1 (Hyr1)

Es una proteína formada por 937 aminoácidos descrita por primera vez por Bailey y colaboradores en 1996.

En su extremidad N-terminal presenta una secuencia señal, mientras que en la extremidad C-terminal presenta un punto de anclaje GPI. Además, la secuencia contiene 17 potenciales sitios de glicosilación y varios dominios característicos, como el dominio hidrofílico (residuos 346-576) rico en serina y treonina, y el dominio rico en asparagina, serina y glicina (residuos 577-816). Aunque el gen se expresa durante la formación de los tubos germinales, cepas mutantes para el gen *HYR1* ($\Delta hyr1/\Delta hyr1$) no mostraban ningún cambio significativo a nivel morfológico en comparación con una cepa salvaje (Bailey et al., 1996).

Esta proteína es un factor muy importante en la virulencia de *C. albicans* generando resistencia a la fagocitosis por parte de los neutrófilos (Luo et al., 2010). En este sentido, se han llevado a cabo estudios desde un punto de vista terapéutico. Luo y colaboradores (Luo, Ibrahim, French, Edwards Jr, & Fu, 2011; Luo et al., 2010) estudiaron el rol protector de la proteína recombinante rHyr1p. Observaron un aumento la tasa de supervivencia y una disminución de la carga fúngica en un modelo murino de CI.

1.5.1.3 Hyphal wall protein (Hwp1)

La Hwp1, descrita por primera vez por Staab y colaboradores en 1996 (Staab, Ferrer, & Sundstrom, 1996), es una adhesina de 634 aminoácidos que se expresa únicamente durante la fase micelial de *C. albicans*. La extremidad N-terminal de esta proteína está involucrada en el proceso de adhesión, presenta una región central rica en serina y treonina sujeta a N y O-glicosilación. Su extremidad C-terminal es sitio de anclaje GPI (Staab, Bahn, Tai, Cook, & Sundstrom, 2004). Juega un papel muy importante en la virulencia de *C. albicans* siendo sustrato de las transaminasas de las células del hospedador (Staab et al., 1999).

La pérdida del gen *HWP1* se demuestra en la falta de adhesión a células epiteliales y reducción de virulencia en modelos animales de CI y en las mucosas (Staab et al.,



1999; Sundstrom, 2002). En este sentido cepas mutantes para el gen *HWP1* ($\Delta hwp1/\Delta hwp1$) mostraron una menor virulencia en comparación con cepas salvajes en un modelo de ratón de CI (Tsuchimori et al., 2000). Además la adhesión mediada por la Hpw1 también contribuye a la formación de biopelículas (Ene & Bennett, 2009).

1.5.2 Factores de adhesión

La adhesión de *Candida* a las células del huésped es un requerimiento esencial durante las primeras fases de colonización del tejido humano, al mismo tiempo esta relación permite el desarrollo de una infección (Silva et al., 2012). Este aspecto está reforzado por diferentes estudios que vieron como cepas avirulentas del hongo *Candida* no eran capaces de adherirse a la célula huésped (Calderone & Braun, 1991; Murciano et al., 2012; Oh et al., 2005; Phan et al., 2007; Zhao et al., 2004). El principal punto de anclaje de *Candida* a las células humanas o a los distintos dispositivos es mediante unas proteínas de la superficie de la pared del hongo denominadas adhesinas (Calderone & Fonzi, 2001; Gonçalves et al., 2016; Murciano et al., 2012; Swidergall & Filler, 2017; Verstrepen & Klis, 2006). Estas proteínas son capaces de reconocer distintos componentes entre las proteínas séricas, componentes de la matriz (laminina, fibronectina, colágeno, vitronectina y entactina) o también promover uniones a superficies abióticas mediante interacciones hidrofóbicas (Chaffin, 2008).

La familia de proteínas de *Candida* más estudiada es la de Agglutinine like sequence (ALS). Esta incluye ocho proteínas, Als1, Als2, Als3, Als4, Als5, Als6, Als7, Als9 (Zordan & Cormack, 2012).

Estas codifican para el glicosilfosfatidilinositol (GPI) asociado a la superficie celular de las glicoproteínas. De todas ellas, la más importante es la Als3, cuya sobreexpresión se ha observado *in vitro* con células epiteliales orales y durante infección vaginal *in vivo* (Cheng et al., 2005; Mayer, Wilson, & Hube, 2013; Murciano et al., 2012).

En este gran grupo de adhesinas se incluyen también los miembros de la familia de la hyphal wall protein (Hwp1) (Sundstrom, 2002; Zordan & Cormack, 2012). Esta sirve de sustrato para las transglutaminasas de mamíferos y su actividad podría ser



necesaria para la unión de *C albicans* a las células de huésped catalizando la formación de enlaces isopeptídicos. Se ha visto como cepas mutantes del gen *HWP1* (*hwp1Δ/Δ*) presentan una capacidad de adhesión reducida y una menor virulencia en un modelo murino de CI (Mayer et al., 2013; Staab et al., 1999; Sundstrom, 2002).

La familia de proteínas de adherencia aumentada a poliestireno (EAP1) (Zordan & Cormack, 2012), derivan de un gen que codifica sitios de unión al glicosilfosfatidilinositol para proteínas de la pared, que son puntos de unión al glucano, e interfiere en la adhesión y formación de biopelículas *in vivo* e *in vitro*. Se ha visto que cepas mutantes del gen *EAP1* presentan adhesión y formación de biopelículas reducida a poliestireno y células epiteliales (Li et al., 2007).

Otros genes que codifican adhesinas son *INT1* (integrin-like) y *MNT1* (α -1,2 manosil transferasa). La proteína Int1 facilita la filamentación, adhesión a las células epiteliales y contribuye a la virulencia en un modelo murino de CI (Kinneberg et al., 1999). Se ha visto que cepas mutantes de *C. albicans* para el gen *INT1* eran menos virulentas, presentaban un 40% menos en la adherencia a células epiteliales, así como una reducción en la filamentación (Calderone & Fonzi, 2001). Por otro lado, el gen *MNT1*, codifica una manosil transferasa que añade residuos de manosa a la pared celular del hongo mediante enlaces O y N-glicosídicos. Las cepas mutantes en este gen presentan una reducción en la capacidad de adhesión a células epiteliales y en su virulencia (Buurman et al., 1998; Calderone & Fonzi, 2001).

Entre los factores de adhesión también hay que incluir componentes de la pared celular del hongo, que no son adhesinas, pero que de alguna forma ayudan y/o participan en el proceso de adhesión del hongo. Es el caso de las fimbrias, mediante las cuales la levadura se une a los glicofosfolípidos que se presentan en la superficie de las células epiteliales orales (Vitkov, Krautgartner, Hannig, Weitgasser, & Stoiber, 2002). También se han identificado polisacáridos (Fukazawa & Kagaya, 1997) y lípidos (Chaffin, 2008) que se encuentran en la pared celular de hongos y que juegan un papel importante en el proceso de adhesión.



1.5.3 Dimorfismo

El dimorfismo es el cambio de un morfotipo levaduriforme unicelular, a uno filamentoso. En este sentido *C. albicans* tiene la capacidad de cambiar reversiblemente su morfología de blastospora a una forma filamentosa (Calderone & Fonzi, 2001). Este cambio morfológico viene estimulado por una serie de factores como una temperatura de 37°C, un pH mayor o igual a 7 y una concentración de CO₂ de 5,5% en presencia de suero o fuentes de carbono. Por otro lado, las levaduras suelen crecer a temperaturas más bajas, con pH más ácidos (pH < 6) y fuentes de glucosa (Mayer et al., 2013).

Se ha visto que cepas mutantes incapaces de formar micelios presentan generalmente una virulencia atenuada en comparación con cepas capaces de germinar (Braun & Johnson, 1997; Braun et al., 2000; Lo et al., 1997). En un estudio genómico a gran escala con 177 cepas mutantes que presentaban defectos en la filamentación, se estableció una correlación con una virulencia atenuada o incluso avirulencia (O'Meara et al., 2015). La mayoría de las cepas que eran capaces de filamentar poseían un alto grado de virulencia, mientras que las que presentaban una virulencia atenuada tenían graves defectos en el crecimiento (Kadosh, 2017).

Aún y todo hay que tener en cuenta que la relación entre virulencia y la capacidad que tiene *Candida* para filamentar es mucho más compleja y que el morfotipo filamentoso no es imprescindible en determinados aspectos de la patogenicidad. En este sentido, aunque ya hay evidencias de que la forma filamentosa es más invasiva que la forma levaduriforme, ambos morfotipos son necesarios en una CI (Jacobsen et al., 2012; Mayer et al., 2013). Dependiendo del órgano infectado se pueden observar una morfología u otra. Por ejemplo, se pueden observar micelios de *C. albicans* en infecciones invasoras en el riñón, pero no en el bazo o en el hígado. Aunque no haya ninguna evidencia clara de la predominancia de un morfotipo u otro implicado en el comensalismo del hongo, se ha propuesto que la fase levaduriforme, por su diminuta forma ovoide (3-9 x 2-8 µm), es fundamental para la diseminación en sangre (Jacobsen et al., 2012).



1.5.4 Cambio fenotípico o *switching*

En 1985 Pomes y colaboradores descubrieron que bajas dosis de rayos UV provocaban la formación de colonias rugosas de *C. albicans* con una frecuencia muy elevada (3×10^{-3}) y que este cambio fenotípico revertía con una frecuencia de 9×10^{-4} (Pomés, Gil, & Nombela, 1985). Este cambio o variante fenotípica viene transmitido de una generación a otra a partir de un único progenitor ancestral. De esta forma, las generaciones sucesivas pueden expresar fenotipos muy diferentes y transmitir estos cambios a las células hijas. En este sentido, cabe destacar que el cambio de blastospora a micelio, no se considera *switching* fenotípico en cuanto se ven involucrados todos los miembros de la población en respuesta a distintos factores ambientales.

Los ejemplos más conocidos de cambio fenotípico son el blanco-opaco, fenómeno en el que células de *C. albicans* pasan de ser blancas con una forma ovalada a una forma más alargada y opacas (Gonçalves et al., 2016). Este *switching* se describió por Anderson y colaboradores en 1987 (Anderson & Soll, 1987). También se han descrito liso-rugoso (Pomés et al., 1985) y liso-micelial (Soll, Langtimm, McDowell, Hicks, & Galask, 1987).

Se ha observado que este cambio fenotípico es capaz de modular determinados factores de virulencia incluyendo la morfología (Vargas et al., 2000), adhesión y secreción de Saps (proteasas aspárticas secretadas) (Morrow, Srikantha, & Soll, 1992), todo ello para incrementar la supervivencia de *Candida* en el lugar de infección y generar un proceso de patogénesis (Gonçalves et al., 2016).

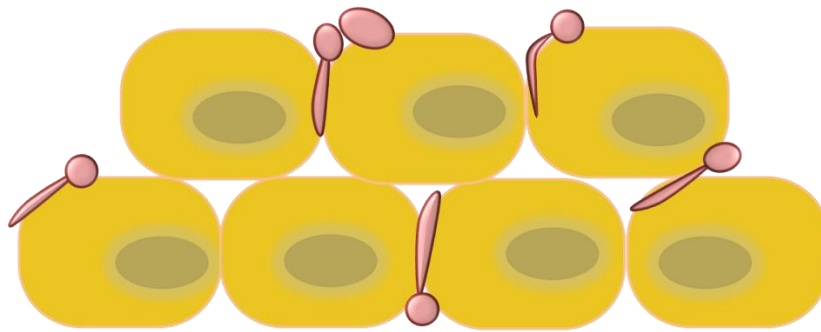
1.5.5 Hidrofobicidad

Se ha demostrado que la hidrofobicidad juega un papel muy importante en la virulencia de *C. albicans* (Hazen, 1989), y está directamente relacionada con la estructura de la pared de *Candida* (Masuoka & Hazen, 1997). Las células hidrofóbicas se adhieren mejor a una variedad de tejidos que las células hidrofílicas siendo al mismo tiempo más resistentes a la fagocitosis por parte de los macrófagos (Hazen, Mandell, Coleman, & Wu, 2000).



1.5.6 *Quorum sensing* y tigmotropismo

El *Quorum sensing* describe un amplio conjunto de fenómenos en los cuales las células se comunican entre sí mediante un sistema de señalización celular llevado a cabo por los microorganismos. Está basado en la producción de moléculas de señalización extracelular que median con el ambiente químico físico y con otros microorganismos (Keller & Surette, 2006). En este sentido el *Quorum sensing* regula funciones tan diferentes como la bioluminiscencia y la virulencia, y es fundamental en los inicios de asentamiento de una biopelícula de *C. albicans* (Finkel & Mitchell, 2012). Por otro lado, las hifas de *C. albicans* presentan un crecimiento direccional en presencia de surcos, hendiduras, poros y otras irregularidades de la superficie con las que está en contacto; a este fenómeno se le llama tigmotropismo (Mayer et al., 2013) (Figura/Figure 1.4), y facilita la invasión y penetración de los micelios de *C. albicans* en los tejidos del huésped (Brand & Gow, 2009).



Figura/Figure 1.4 Esquema representando tigmotropismo en *C. albicans*.

1.5.7 Enzimas hidrolíticos

El hongo *Candida* es capaz de secretar una serie de enzimas hidrolíticos que juegan un papel muy importante en la adhesión, penetración de tejidos, invasión y degradación de tejidos del huésped (Schaller, Borelli, Korting, & Hube, 2005). Estos enzimas están implicados frecuentemente en la patogenicidad y virulencia del hongo (Calderone & Fonzi, 2001; Gonçalves et al., 2016). En este grupo, se han descrito las aspartil proteinasas (Sap), fosfolipasas y lipasas (Gonçalves et al., 2016; Naglik et al., 2003; Silva et al., 2012).



Las proteinasas aspárticas facilitan la adhesión al tejido del huésped, provocan daño celular y están relacionadas con la respuesta inmune del hospedador (Pichová et al., 2001). Hasta hoy en día se han identificado diez genes *SAP* (*SAP1-10*) en *C. albicans* (Odds, 1988). Estas proteínas actúan en condiciones muy ácidas ($\text{pH} \leq 4$) (Mayer et al., 2013; Williams, Kuriyama, Silva, Malic, & Lewis, 2011). De todas ellas, Sap1-8 son secretadas al medio, mientras que Sap9 y Sap10 permanecen ancladas en la superficie celular (Albrecht et al., 2006; Naglik et al., 2003; Taylor et al., 2005). También se ha visto que Sap1-3 son necesarias para generar daño a nivel del tejido epitelial *in vitro* así como para la virulencia en un modelo murino de CI (Correia et al., 2010; Lermann & Morschhäuser, 2008).

La familia de las fosfolipasas está compuesta por cuatro clases diferentes (A, B, C y D) (Niewerth & Korting, 2001). De estas tan sólo cinco componentes de la clase B (fosfolipasa B; PLB1-5) son extracelulares y están implicados en la patogenicidad del hongo (Mavor, Thewes, & Hube, 2005). Se ha visto que cepas mutantes de *plb1Δ/Δ* y *plb5Δ/Δ* presentan una virulencia atenuada en un modelo murino de CI (Leidich et al., 1998; Mayer et al., 2013; Theiss et al., 2006). Las fosfolipasas actúan hidrolizando uno o más enlaces éster de los glicerofosfolípidos contribuyendo al daño celular y a la adhesión de las levaduras en el tejido del huésped (Gonçalves et al., 2016).

El grupo de las lipasas está codificado por diez genes (*LIP1-10*) (Silva et al., 2012). Estas actúan hidrolizando los triglicéridos y su actividad se ha asociado a la adhesión y daño tisular (Stehr et al., 2004). También se ha visto su asociación con la inhibición de la quimiotaxis mediada por células así como con la producción de daño en los fagocitos (Schaller et al., 2005).

1.5.8 Adquisición de hierro

El hierro es vital tanto para el hospedador como para *C. albicans*, su capacidad para absorberlo se considera un factor de virulencia a lo largo de la colonización e invasión, siendo necesario a lo largo de todo el proceso de patogénesis (Sutak, Lesuisse, Tachezy, & Richardson, 2008). Además, el hierro es un cofactor esencial para diferentes proteínas y es necesario para numerosos procesos bioquímicos como la respiración celular, transporte de oxígeno, metabolización de fármacos y



síntesis de ADN (Welch, Van Eden, & Aust, 2001). Por lo tanto, un aumento de la concentración de hierro en la sangre aumenta la susceptibilidad del hospedador a las infecciones generadas por *C. albicans* (Almeida, Wilson, & Hube, 2009). Por el contrario, la falta de hierro altera la composición de la superficie celular e inhibe la formación de los tubos germinales (Lan et al., 2004). *C. albicans* posee tres importantes mecanismos para obtener el hierro: el primero es un sistema reductor de adquisición del hierro a partir de la ferritina y transferrina del huésped o del medio ambiente, el segundo es un sistema de sideróforos producidos por otros organismos (xeno-sideróforos), y en tercer lugar mediante un sistema de adquisición a partir de hemoglobina y proteínas hemáticas del huésped (Almeida et al., 2009).

1.5.9 Mimetismo molecular

El mimetismo molecular es la capacidad que tiene un organismo para expresar en su superficie moléculas similares a las del huésped. De este modo, es capaz de evadir el sistema inmune y por lo tanto evitar su eliminación. Ejemplo de ello son las blastosporas de *C. albicans* que expresan en su superficie una proteína muy similar a la CD11b/CD18, un miembro de la integrina $\beta 2$, clasificada como una adhesina de los neutrófilos de mamíferos. Ambas proteínas presenta afinidad para el ligando C3iC3b y se sobreexpresan a 37°C (Gustafson, Vercellotti, Bendel, & Hostetter, 1991), lo que permite al hongo mimetizarse frente a los neutrófilos y evadir de esta forma su fagocitosis.

1.5.10 Sinergismo con otros microorganismos

C. albicans pertenece a la microbiota normal de las mucosas humanas y por lo tanto comparte el mismo nicho ecológico con otras especies, por ejemplo, anaerobias y estreptococos, estando obligada a compartir y/o competir por los mismos recursos. Debido a que tanto las bacterias como *Candida* comparten los mismos receptores epiteliales, las bacterias modulan la colonización por parte del hongo en las superficies mucosas (Kennedy & Volz, 1985), incluyendo el tracto gastrointestinal (Lopez-Medina & Koh, 2016).

La relación que presenta *Candida* con otros microorganismos, no es solamente antagonica, sino también sinérgica. En este sentido, *C. albicans* es capaz de



establecer una relación con las bacterias patógenas *Staphylococcus aureus* o *Pseudomonas aeruginosa* (Fourie et al., 2016), formando biopelículas, que le confieren resistencia a determinados antifúngicos (Kean et al., 2017).

1.5.11 Respuesta al estrés

C. albicans presenta una eficaz respuesta al estrés que facilita su adaptación a diferentes condiciones ambientales, protegiendo al hongo frente a los mecanismos de defensa del huésped y contribuyendo a la virulencia de *Candida*. Las diferentes respuestas celulares incluyen una respuesta a estrés térmico, osmótico, oxidativo y nitrosativo (Mayer et al., 2013).

La respuesta al estrés térmico está mediada por las proteínas de choque térmico (HSP, Heat Shock Protein) que actúan como chaperonas para prevenir plegamientos y agregaciones de las proteínas. El estrés osmótico se combate mediante la acumulación intracelular de diferentes solutos como el glicerol, para contrarrestar la pérdida de agua debida al gradiente químico. La biosíntesis del glicerol está mediada por la glicerol-3-fosfatasa (Gpp1) y por la glicerol-3-fosfato deshidrogenasa (Gpd1); se ha visto que cepas mutantes de estos genes (*gpp1Δ/Δ* y *gpd2Δ/Δ*) presentaban una capacidad reducida para dañar células epiteliales *in vitro*, así como defectos en el crecimiento (Fan, Whiteway, & Shen, 2005; Mayer et al., 2013). Por otro lado, el estrés oxidativo está inducido por especies reactivas al oxígeno (ROS) como el peróxido, aniones superóxido y radicales hidroxilos. La catalasa Cta1 y las superóxido dismutasas (Sod1 y Sod2) son cruciales para la detoxificación de las ROS (Hwang et al., 2002; Martchenko, Alarco, Harcus, & Whiteway, 2004; Mayer et al., 2013; Wysong, Christin, Sugar, Robbins, & Diamond, 1998). El estrés nitrosativo está inducido por los neutrófilos que producen especies reactivas al nitrógeno (RNS) contra las células fagocitadas de *C. albicans*. La proteína con mayor implicación en este tipo de respuesta es la flavohemoglobina Yhb1. Se ha observado que cepas de *C. albicans* defectivas para estas proteínas son sensibles a las RNS y presentan virulencia atenuada en un modelo animal de CI (Hromatka, Noble, & Johnson, 2005).



1.5.12 Formación de biopelículas (*biofilms*)

La capacidad de formar biopelículas, en superficies biológicas o inertes, es uno de los factores de virulencia más importantes de *C. albicans*. Los sustratos más comunes son catéteres, prótesis dentales y mucosas (Fanning & Mitchell, 2012). La formación de una biopelícula se produce mediante una serie secuencial de eventos que incluyen la adhesión de las levaduras al sustrato, la proliferación de dichas levaduras, la formación de hifas en la parte superior de la biopelícula, la acumulación de material extracelular y finalmente la dispersión de las levaduras de este complejo. Las biopelículas son mucho más resistentes a los antimicrobianos que las células planctónicas (Fanning & Mitchell, 2012; Uppuluri & Ribot, 2017). Esto se debe a su compleja arquitectura, a su matriz, a la sobreexpresión de bombas que expulsan determinados fármacos y a su plasticidad metabólica (Fanning & Mitchell, 2012; Mayer et al., 2013). La dispersión de blastosporas de una biopelícula está directamente relacionada con su virulencia en un modelo murino de CI (Uppuluri et al., 2010). En este sentido, la proteína de choque térmico Hsp90 es clave en la dispersión de blastosporas de *C. albicans* de biopelículas. Además, la misma proteína es necesaria para el aumento de la resistencia a fármacos antifúngicos (Robbins et al., 2011).

1.6 Características antigénicas de *Candida*

El estudio de las características antigénicas de *Candida* presenta un gran interés tanto desde el punto de vista del diagnóstico de la CI como terapéutico. La detección de anticuerpos anti-*Candida*, así como la detección de componentes antigénicos del hongo, se han visto de gran utilidad para el diagnóstico de la CI. También se ha observado como algunos anticuerpos dirigidos frente componentes de los tubos germinales de *C. albicans* pueden tener un efecto inmunoprotector (Iruretagoyena, Regúlez, Quindós, & Pontón, 2006; Zaragoza et al., 2009a), abriendo camino al desarrollo de vacunas como estrategia para tratar y prevenir esta enfermedad (Huertas et al., 2016; Lopez-Ribot, Casanova, Murgui, & Martinez, 2004; Moragues, Rementeria, Sevilla, Eraso, & Quindos, 2014).

La composición antigénica de *C. albicans* es muy compleja y, a medida que se van empleando métodos más sofisticados de identificación, se están descubriendo cada



vez más antígenos. En este sentido, las técnicas inmunoproteómicas usando sueros de pacientes infectados con CI permiten detectar aquellos antígenos reconocidos por los pacientes infectados. Los sueros se pueden ensayar frente a extractos de tubos germinales de *C. albicans* o frente proteínas generadas a partir de una genoteca de expresión (phage display) de *Candida* en fase filamentosa, pudiendo aislar y reconocer los clones positivos. Estos avances permitirían el descubrimiento de nuevos biomarcadores de la CI que pueden ser utilizados desde un punto de vista diagnóstico y terapéutico (Pitarch, Nombela, & Gil, 2010; Wu, Liu, Lu, & Wu, 2016).

Estos antígenos forman parte de las distintas estructuras de *Candida* y se encuentran localizados básicamente en tres compartimentos distintos: el citoplasma, la pared celular y los antígenos secretados al medio.

1.6.1 Antígenos citoplasmáticos

El estudio de los antígenos citoplasmáticos fue impulsado por la teoría de que para su liberación era necesaria la destrucción del hongo por las células fagocíticas del paciente y, por tanto, la sensibilización frente a estos antígenos se produciría solamente durante la invasión tisular.

Mediante técnicas de inmunoproteómica se han identificado un total de 42 antígenos citoplasmáticos de *C. albicans*, cuya mayoría son proteínas *housekeeping*, es decir proteínas necesarias para las funciones básicas de la célula. Estos se pueden agrupar en diferentes categorías dependiendo de su función (Pitarch, Jiménez, Nombela, & Gil, 2008):

1. Proteínas de choque térmico (Hsp90 y miembros de la familia de la Hsp70 como la Ssa1, proteína Ssb1, Ssc1 y Sse1) y proteínas disulfuro isomerasa (Pdi1);
2. Proteínas implicadas en diferentes rutas metabólicas:
 - Carbohidratos: se incluyen todas las enzimas de la ruta glicolítica altamente conservada como la fosfofructosaquinasa (Pfk1), hexoquinasa-2 (Hxk2), fosfoglucoisomerasa-1 (Pgi1), fructosa-1,6-bisfosfato aldolasa (Fba1), triosafosfato isomerasa-1 (Tpi1), gliceraldehído-3-fosfato deshidrogenasa (Gap1), fosfoglicerato quinasa-1 (Pgk1); enzimas fermentativos como piruvato descarboxilasa (Pdc11) y



- Adh, enzimas del ciclo de los ácido tricarboxílicos como aconitasa (Aco1) y malato deshidrogenasa (Mdh1), y otras como transcetolasa (Tkl1), inositol-1-fosfato sintasa (Ino1) y acetil-CoA sintasa (Acs2)
- Ácidos grasos: acetil-CoA-hidrolasa (Ach1)
 - Aminoácidos: metionina sintasa (Met-6), S-adenosil-L-homocisteína hidrolasa (Sah1), cetoácido reductoisomerasa (Ilv5), serina hidrometiltransferasa (Shm2) e isopropil malato deshidratasa (Leu1)
 - Nucleótidos: inosin monofosfato deshidrogenasa (Imh3) y adenosin-imidazol-carboxamida-ribonucleótido transfromilasa (Ade17)
 - Energía: ubiquinol-citocromo-c reductasa (Qcr2), subunidad beta F1 del complejo F1F0-ATPasa (Atp2) y pirofosfatasa
 - Factores de elongación (Eft3, Eft2, Tif1) y proteínas ribosómicas (Bel1)
 - Porinas: porina-1 (Por1)
 - Enzimas redox: metilglioxal-reductasa (Grp1) y coproporfirinógeno oxidasa (Hem13)
 - Proteínas de función desconocida como la Ipf1786 o manoproteínas de 50, 51 y 52 KDa.

Estos mismos autores, mediante estudios de geles 2D, identificaron 15 proteínas en el extracto citoplasmático de levaduras de *C. albicans* que eran reconocidas por IgG de pacientes con CI, en comparación con un grupo control sin infección. Estas eran las proteínas de choque térmico Ssa y Ssc, Fba1, Pgc1, Eno-1, Pdc1, Adh1, Aco1, Mdh1, Met-6, Ade1, Eft3, la proteína ribosomal Bel1, Grp2 e Ipf7186 (Pitarch et al., 2008).

Hernando y colaboradores (Hernando et al., 2010) ensayaron extractos citoplasmáticos de micelios de *C. albicans* con la misma metodología de Pitarch y colaboradores (Pitarch et al., 2008). En este caso detectaron 16 antígenos, de los cuales 10 aparecían tanto en el extracto citoplasmático como en el de los micelios. De los antígenos restantes, 4 se observaron solo en las blastosporas y 2 en los micelios. De las 8 proteínas que se identificaron, Eno-1, Pgc1, Mdh1 y dos isoformas de Fba ya estaban descritas (Pitarch et al., 2004), mientras que el precursor de la Kexina, la chaperona I del complejo mitocondrial y un dominio catalítico de la diacilglicerol quinasa fueron descritas por primera vez en este estudio.



En un estudio proteómico de extractos de pared celular de blastosporas y tubos germinales de *C. albicans*, Saéz-Rosón y colaboradores identificaron 7 proteínas [Met-6, Ino1, Eno-1, Adh1, Pgc1, proteína 14-3-3 (Bmh1) y la subunidad alfa del complejo asociado al polipéptido naciente (Egd2)] que eran reconocidas por los anticuerpos anti-micelio (CAGTA) desarrollados en un modelo de candidiasis invasora en conejo (Sáez-Rosón, Sevilla, & Moragues, 2014), algunas de las cuales coincidían con las descritas previamente por otros autores en los extractos citoplasmáticos. La Enolasa (Eno-1) es una de los antígenos más relevantes de *C. albicans* tanto en el campo diagnóstico como en inmunoterapia. Esta enzima (2-fosfato-D-glicerato-hidrolasa) de 440 aminoácidos está codificada por un solo gen, *ENO1*, y su secuencia de aminoácidos presenta un alto grado de homología con las enolasas 1 y 2 de *S. cerevisiae*. Cataliza la reacción reversible de 2-fosfo-D-glicerato a fosfoenolpiruvato de la ruta de la glucólisis y la gluconeogénesis. Puede actuar también como alérgeno (Ishiguro, Homma, Torii, & Tanaka, 1992) y como receptor del plasminógeno humano generando una enzima proteolítica, la plasmina, aumentando la capacidad de invasión tisular y necrosis del hongo (Jong et al., 2003). Aunque la Eno-1 es una enzima mayoritariamente citoplasmática (Ebanks, Chisholm, McKinnon, Whiteway, & Pinto, 2006; Hernáez et al., 2010; Martínez-Gomariz et al., 2009; Monteoliva et al., 2010; Pitarch, Sanchez, Nombela, & Gil, 2002; Sáez-Rosón et al., 2014), se puede encontrar en el sobrenadante de cultivos de *C. albicans* (Sundstrom & Aliaga, 1994) y en el torrente sanguíneo de pacientes con CI (Walsh et al., 1991).

En pacientes con CI se han detectado anticuerpos específicos frente a enolasa, demostrando su naturaleza inmunogénica y su utilidad en el diagnóstico (Laín et al., 2007b; Philip et al., 2005; Pitarch et al., 2008), así como su posible papel inmunoprotector frente al desarrollo de una Candidiasis Invasora (Pitarch, Jimenez, Nombela, & Gil, 2006). En este sentido se observó un efecto protector tanto en modelos murinos como en estudios con pacientes con CI. Van Deventer y colaboradores (van Deventer, van Vliet, Hop, & Goessens, 1994) comprobaron que la inmunización pasiva de ratones con estos anticuerpos disminuía la tasa de mortalidad de los animales infectados con *C. albicans*. En esta línea, Montagnoli y colaboradores (Montagnoli, Sandini, Bacci, Romani, & Valle, 2004) observaron que ratones inmunizados con la proteína recombinante Eno-1 junto con interleukina12



(IL-12), y posteriormente infectados con *C. albicans*, presentaban una mayor tasa de supervivencia y una carga fúngica inferior en los riñones en comparación con ratones no inmunizados. Más recientemente, Li y colaboradores consiguieron resultados similares (Li et al., 2013).

1.6.2 Antígenos de la pared celular

La pared celular de *C. albicans* es la estructura más externa, y contiene un gran número de antígenos capaces de estimular una respuesta inmunológica importante. Estos antígenos son de gran interés ya que están involucrados en distintos aspectos de la patogenicidad de *C. albicans*, tales como la adhesión, antigenicidad, hidrofobicidad, inmunomodulación, reconocimiento por el sistema inmune del huésped y virulencia (Chaffin, 2008; Pitarch et al., 2006). Debido a su fácil accesibilidad y extracción han sido los más estudiados de *C. albicans*, y pueden ser de naturaleza glucídica o proteica.

Los principales antígenos de naturaleza glucídica son el β -glucano y el manano, y ambos forman parte de importantes líneas de investigación en el diagnóstico de la CI. En cuanto a los antígenos de naturaleza proteica, Pitarch y colaboradores (Pitarch et al., 2006) mostraron la capacidad inmunogénica de algunos de ellos como Bgl2, Eno-1, Tpi1, Pkg1, Met-6 y Gap1 (Moragues et al., 2014). Por otro lado, también se ha descrito el papel inmunoprotector de anticuerpos específicos contra antígenos de pared como el manano y β -glucano (De Bernardis et al., 1997; Lopez-Ribot et al., 2004) y proteínas como Als1, Als3, Hsp90, Hyr1 y Hwp1 (Moragues et al., 2014) abriendo camino a su utilidad en el desarrollo de una posible vacuna.

1.6.3 Antígenos secretados al medio

Al igual que otros microorganismos, *C. albicans* secreta al medio una serie de componentes, algunos de los cuales son antigénicos. Entre ellos destacan las proteasas aspárticas (SAPs) y las fosfolipasas, en especial la fosfolipasa B. Estas proteínas han sido ampliamente estudiadas debido a que juegan un papel muy importante en la virulencia y patogenicidad del hongo, como ya se ha descrito previamente en el apartado 1.5.7.



Las proteínas Sap con sus respectivos anticuerpos se han estudiado como posibles candidatos a vacunas. En este sentido la casa comercial Pevion Biotech AG (Ittigen, Suiza) está realizando los correspondientes ensayos clínicos de la vacuna intramuscular PEV7, basada en una proteína Sap2 recombinante, frente a la candidiasis vulvovaginal (Edwards Jr, 2012).

1.7 Diagnóstico de la CI

1.7.1 Diagnóstico microbiológico convencional

El examen directo es fundamental para el diagnóstico de infección por *Candida* en candidiasis superficiales tales como las candidiasis orales, esofágicas, balanitis candidiásica o vulvovaginitis. Puede efectuarse un examen en fresco con KOH o utilizando tinciones como la del blanco de Calcofluor y observar blastoconidias redondas, alargadas u ovaladas. También se estudian biopsias aprovechando tinciones como la del ácido periódico-Schiff (PAS). Pero estas técnicas solo permiten revelar estructuras fúngicas levaduriformes o filamentosas, sin poder llegar a su identificación. La utilización de otros fluorocromos como el Blankophor P y el Rojo Congo permite aumentar la sensibilidad (Llovo & Pontón, 2007).

Por el contrario, en candidiasis sistémicas, como método de rutina en los laboratorios las muestras se inoculan en medios de cultivo que requieren periodos de incubación entre 24 y 48 horas para el aislamiento de colonias. Entre los medios de cultivo más utilizados están el agar Sabouraud, agar sangre y medios cromógenos diferenciales como CHROMagar Candida®, aunque este último tiene una sensibilidad inferior. En el caso de los hemocultivos, se ha mejorado mucho en los últimos años consiguiendo llegar a valores de sensibilidad entre el 60% y el 70% (de Bedout & Gómez, 2010).

Recientemente se han ido produciendo grandes avances en el desarrollo de nuevas técnicas comerciales más rápidas y fiables, destacando el sistema MALDI-TOF. Este sistema de espectrometría de masas permite identificar un elevado número de microorganismos patógenos a partir del hemocultivo, convirtiéndose en una herramienta importante en los laboratorios clínicos (Bao et al., 2018; Croxatto, Prod'hom, & Greub, 2012; Turhan et al., 2017). Yaman y colaboradores (Yaman, Akyar, & Can, 2012) comprobaron que el sistema MALDI-TOF fue capaz de



identificar el 100% de las especies de *Candida* en un total de 281 muestras y sobre todo, las que más comúnmente provocan una CI como *C. albicans*, *C. tropicalis*, *C. glabrata*, *C. parapsilosis*, *C. guilliermondii* y *C. krusei*.

Por otro lado, el diagnóstico mediante cultivo puede presentar problemas de especificidad, ya que *Candida* es un colonizador habitual de las mucosas humanas y puede dar un resultado falso positivo en individuos que son simplemente portadores. Por tanto, debido a los problemas de sensibilidad y especificidad de las técnicas disponibles en la actualidad, en muchas ocasiones la confirmación de una CI se obtiene solo en las fases avanzadas de la enfermedad con el resultado de una mortalidad y morbilidad significativa en pacientes críticos.

1.7.2 Diagnóstico microbiológico independiente del cultivo

1.7.2.1 Pruebas serológicas

En las últimas décadas se ha visto reforzada la investigación para el desarrollo de pruebas serológicas basadas en nuevos marcadores para el diagnóstico temprano de la CI. Estas técnicas se basan en la detección de antígenos y anticuerpos mediante los métodos serológicos de ELISA (acrónimo del inglés Enzyme-Linked Immunosorbent Assay) y la inmunofluorescencia indirecta (IFI).

1.7.2.1.1 Detección de anticuerpos

La detección de anticuerpos puede estar limitada por la presencia de falsos positivos en individuos colonizados, y falsos negativos en individuos inmunodeprimidos que no son capaces de producir niveles suficientes de anticuerpos. Aún y todo, se ha considerado que su detección pueda compensar la elevada transitoriedad de los antígenos.

La enolasa, induce una importante respuesta inmune, por lo que la detección de anticuerpos anti-enolasa ha sido un reto fundamental en el diagnóstico de la CI. Van Deventer y colaboradores (van Deventer et al., 1994) utilizando la enolasa purificada para el diagnóstico de la CI obtuvieron resultados no muy satisfactorios con valores de sensibilidad (S) del 50% y una especificidad (E) del 78%.

Se han desarrollado kits comerciales como SysCan3® (Rockeby Biomed Ltd., Australia) y *Candida* Enolasa ELISA Immunoglobulin G (IgG) (Laboratorios Vircell,



Granada, España) para detectar anticuerpos anti-enolasa de *Candida* mediante ELISA (Lain et al., 2007b).

El manano también induce una importante respuesta inmunológica, pero los anticuerpos anti-manano son ubicuos en el ser humano y debido a ello los test desarrollados para la detección de estos anticuerpos no son capaces de diferenciar una colonización de una invasión. Sendid y colaboradores (Sendid et al., 1999) hicieron un estudio combinando la detección del antígeno manano y de los anticuerpos anti-manano, y consiguieron una sensibilidad del 88,9% y una especificidad del 82,6%. Mikulska y colaboradores (Mikulska, Calandra, Sanguinetti, Poulain, & Viscoli, 2010) recientemente, revisaron un número elevado de publicaciones que reportaban el estudio combinado del antígeno manano con los anticuerpos anti-manano, donde registraron un valor medio de S= 62% y E=96%. Duettmann et al. hicieron un estudio combinado de antígenos y anticuerpos anti-manano en pacientes con cáncer hematológico. Observaron una especificidad del 74,8% (Duettmann et al., 2016). Martínez-Jiménez y colaboradores en un estudio combinando de manano y anti-manano de un total de 32 pacientes con CI observaron unos valores de 63,3%, 95,7%, 90%, 81,8% de sensibilidad, especificidad, valor predictivo positivo (VPP) y negativo (VPN) respectivamente para el manano, y de S=61,5%, E=68,5%, VVP=55,2%, VPN=71,4% para el anti-manano. Sus combinaciones dieron una sensibilidad de 86,2, una especificidad de 60,5, un valor predictivo positivo de 62,5 y un valor predictivo negativo 82,2 respectivamente (Martínez-Jiménez et al., 2015).

También se ha estudiado la detección de anticuerpos contra las proteasas aspárticas y la metaloproteína de 52 kDa de *C. albicans* por ELISA. En ambos casos se ha comprobado su utilidad en el diagnóstico de las CI con resultados satisfactorios; en el caso de las proteínas aspárticas, Na y colaboradores observaron una sensibilidad del 93,9% y una especificidad entre el 92 y 96% (Na & Song, 1999). En el caso de la detección de anticuerpos anti-metaloproteína, Moudni y colaboradores consiguieron una sensibilidad del 83% y una especificidad del 76% (El Moudni, Rodier, Daniault, & Jacquemin, 1998), sin embargo, de momento no han tenido ninguna aplicación en la clínica.



1.7.2.1.2 Utilización de proteínas recombinantes como biomarcadores

En los últimos años se han identificado algunos de los antígenos de la pared de los tubos germinales de *C. albicans*, entre ellos destacan Hwp1, Als3, Ece1 y Hyr1. El conocimiento de las secuencias de los genes codificantes de estas proteínas ha permitido, mediante técnicas de ingeniería genética, la obtención de dichas proteínas en forma recombinante y su posterior utilización para la detección de anticuerpos frente a ellas. De este modo, en el laboratorio del Dr. Pontón (UPV-EHU), Laín et al. (Laín et al., 2007a) pusieron a punto un ensayo para la detección de anticuerpos contra el fragmento amino terminal de la Hwp1. La proteína recombinante Hwp1 tiene un dominio N-terminal que se une a los anticuerpos, mientras que el extremo carboxilo está integrado en la pared celular. La detección de anticuerpos contra el fragmento N-terminal de Hwp1 por ELISA mostró unos valores elevados de sensibilidad, especificidad, valores predictivos positivo y negativo de 88,9%, 82,6%, 80,0% y 90,2%, respectivamente, indicando su posible utilidad para el diagnóstico de una CI.

Así mismo, Laín y colaboradores (Laín et al., 2008) también estudiaron la presencia de anticuerpos frente a la proteína recombinante Hyr1 por ELISA en 36 sueros de pacientes con CI y en 45 pacientes del grupo control sin candidiasis. Los valores de sensibilidad y especificidad obtenidos fueron respectivamente 58,3% y 82,2%.

Por el contrario, los ensayos para la detección de anticuerpos contra el segmento N-terminal de la proteína recombinante Als3, o contra la proteína Ece1 han dado resultados poco satisfactorios, mostrando una sensibilidad del 41,7% y una especificidad del 77,8% para la primera, y 55,6% y 80%, respectivamente, para la segunda (Laín et al., 2008).

Por otra parte, los resultados obtenidos mediante ELISA para la detección de anticuerpos anti-enolasa recombinante han dado resultados muy prometedores. En un estudio con 98 pacientes (47 inmunodeprimidos y 51 inmunocompetentes) la utilidad diagnóstica del ensayo fue muy similar en ambos grupos, con unos valores de sensibilidad de 78,9% y 82,6%, y una especificidad del 89,3% y 78,6%, respectivamente (Laín et al., 2007b). Pitarch y colaboradores, obtuvieron unos valores de 67%, 93%, 79% y 88% de sensibilidad, especificidad, valor predictivo positivo y negativo en un total de 163 pacientes de los cuales 45 presentaban una CI



(Pitarch et al., 2008). Mientras He y colaboradores obtuvieron unos valores de sensibilidad, especificidad, valor predictivo positivo y negativo del 88,5%, 90%, 90,2% y 88,2% respectivamente en un total de 102 pacientes de los cuales 52 presentaban una CI (He et al., 2015).

Li y colaboradores, obtuvieron frente la enolasa recombinante unos valores de 72,3% de sensibilidad, 94,7% de especificidad, un 93% de valor predictivo positivo y un 78,5% de valor predictivo negativo. En el mismo estudio se observó la respuesta frente la proteína recombinante Fba1 obteniendo unos valores de 87,1%, 92,8%, 96,4% y 76,5 % de sensibilidad, especificidad, valor predictivo positivo y negativo respectivamente (Li et al., 2013).

Yang y colaboradores desarrollaron un ELISA que detectara la proteína aspártica (Sap2) previamente clonada en el fago pfd88. Obtuvieron unos valores de 60% y 85% de sensibilidad y especificidad respectivamente (Yang et al., 2007).

Estos datos apoyan el papel de la detección de anticuerpos frente a antígenos recombinantes como una herramienta muy útil para el diagnóstico de la candidiasis invasora. Además, la combinación de resultados frente a diferentes antígenos en un mismo ensayo puede contribuir a mejorar la validez diagnóstica de la detección de anticuerpos frente a marcadores específicos.

1.7.2.1.3 Detección de anticuerpos anti-micelio (CAGTA)

Un avance interesante ha sido el desarrollo de la detección de anticuerpos específicos contra la fase micelial de *C. albicans* (CAGTA; *Candida albicans* germ-tube antibodies) mediante inmunofluorescencia indirecta (IFI). En este sentido el grupo de Micología Médica del Dr. Pontón desarrolló una técnica de inmunofluorescencia indirecta que permitió detectar estos anticuerpos anti-micelio diferenciando una colonización por *Candida* de una CI, ya que estos antígenos se expresan en la superficie de la pared celular del micelio, morfología asociada a la invasión tisular. Laboratorios Vircell (Granada, España) ha comercializado el kit diagnóstico *Candida albicans* IFA IgG® basado en esta técnica, la cual presenta una sensibilidad del 84,4% y una especificidad del 94,7% para el diagnóstico de la CI (Moragues et al., 2004). La detección de estos anticuerpos también puede ser útil en el seguimiento de terapias en pacientes con candidiasis invasora diagnosticada (Iruretagoyena et al., 2006). Además, Zaragoza y colaboradores le atribuyen valor pronóstico, ya que pacientes



de UCI con títulos CAGTA en aumento presentan mayores posibilidades de supervivencia (Zaragoza et al., 2009a). Martínez-Jiménez y colaboradores hicieron un estudio con un total de 50 pacientes con una CI y obtuvieron unos valores de especificidad y valor predictivo positivo del 95% (Martínez-Jiménez et al., 2014). Este mismo grupo realizó un segundo estudio con un total de 31 pacientes con CI y observaron un 58,1%, 92%, 81,8% y 78% de sensibilidad, especificidad, valor predictivo positivo y negativo respectivamente (Martínez-Jiménez et al., 2015). La detección de CAGTA es útil para el diagnóstico de una CI no solamente en pacientes infectados por *C. albicans*, sino que también por otras especies de *Candida* como *C. tropicalis* y *C. parapsilosis* (Martínez-Jiménez et al., 2015; Moragues et al., 2004).

1.7.2.1.4 Detección de antígenos

Se han estudiado numerosos antígenos que podrían resultar útiles para el diagnóstico de una CI. Sin embargo, su detección puede presentar bajos niveles de sensibilidad debido al carácter transitorio de su presencia en sangre por la formación de complejos inmunes. Los antígenos estudiados forman parte de la estructura del hongo y se localizan en la pared celular y en el citoplasma.

Los antígenos más importantes de los extractos citoplasmáticos utilizados para el diagnóstico de una CI son la enolasa y un antígeno de 47 kDa, que es un fragmento de una proteína de choque térmico de 90 kDa (Hsp90).

En la pared celular de *Candida* cabe destacar el manano, un polisacárido de alto peso molecular, y en la mayor parte de los estudios utilizan la prueba comercializada Platelia *Candida* Ag® (BioRad) para su detección (Pontón & del Palacio, 2007). Las diferentes especies de *Candida* varían en su contenido en manano y además suelen formar inmunocomplejos con los anticuerpos anti-manano en el suero de los pacientes, que debido a su naturaleza polisacáridica se disocian por la utilización de enzimas proteolíticos o por calentamiento, permitiendo así su detección. Debido a su elevada transitoriedad en la sangre, es muy importante un estudio seriado de diferentes muestras de un mismo paciente para aumentar la posibilidad de detectarlo. Todos estos son aspectos que reducen la sensibilidad de la prueba. La detección de manano (Pastorex *Candida*, BioRad, EEUU) en el diagnóstico de la CI en pacientes críticos dio valores de sensibilidad y especificidad del 52,6% y 100%



respectivamente, mientras que para la Hsp90 los valores fueron del 87,5% (S) y del 92,8% (E) (Pontón, 2006).

Martínez-Jiménez y colaboradores en un estudio realizado con 31 pacientes con CI, en ensayos de detección de antígenos de manano, obtuvieron unos valores de detección de manano del 64,3%, 95,7%, 90%, y 81,8% de sensibilidad, especificidad, valor predictivo positivo y negativo respectivamente (Martínez-Jiménez et al., 2015). Estos valores han mejorado en combinación con la detección de anticuerpos anti-manano como evidenciado en el apartado 1.7.2.1.1. Otro antígeno presente en la pared celular de *C. albicans* es un antígeno de alto peso molecular (> 260 kDa) específico de la fase micelial (de Bedout & Gómez, 2010; Pontón, 2006).

En un estudio retrospectivo para la detección de antígenos de manano de 305 pacientes hospitalizados en el hospital Universitario de Vienna, se utilizaron el Serion ELISA Antigen *Candida* Assay (Serion GmbH, Würzburg, Alemania), Platelia *Candida* Antigen Assay (Bio-Rad Laboratories, Marnes-la-Coquette, Francia), y el más reciente Platelia *Candida* Antigen Plus (Bio-Rad Laboratories, Marnes-la-Coquette, France), se muestran los resultados en la Tabla/Table 1.1 (Hartl et al., 2018).

Tabla/Table 1.1 Valores obtenidos de Sensibilidad (S), especificidad (E), valor predictivo positivo (VPP) y negativo (VPN) de los ensayo de detección de manano realizados: Platelia, Platelia Plus y Serion. Tabla obtenida de (Hartl et al., 2018).

	Platelia		Platelia Plus		Serion	
	CI probadas	CI probadas, posibles o probables	CI probadas	CI probadas, posibles o probables	CI probadas	CI probadas, posibles o probables
S (%)	0.65	0.41	0.85	0.39	0.52	0.37
E (%)	0.98	0.98	0.89	0.89	0.98	0.98
VPP (%)	0.48	0.67	0.26	0.39	0.41	0.60
VPN (%)	0.99	0.95	0.99	0.89	0.98	0.94



1.7.2.2 Detección de componentes no antigénicos

1.7.2.2.1 (1,3)- β -D-glucano

El (1,3)- β -D-glucano es un componente de la pared celular de diferentes especies fúngicas relacionadas con la infección invasora en humanos. Se detecta en pacientes con candidiasis, aspergilosis, neumocistosis y algunos con criptococosis, pero no en las zigomicosis. Dado que es un marcador panfúngico, su detección debe de acompañarse con otras técnicas que permitan determinar el hongo implicado; así en la candidiasis invasora esta técnica podría complementarse con la detección de CAGTA. La técnica de detección del (1,3)- β -D-glucano ha sido incluida como criterio de diagnóstico de las enfermedades fúngicas invasoras (De Pauw et al., 2008). Existen varias pruebas comerciales para su detección: Fungitec-G test (Seikagaku Corporation, Tokio, Japón) presenta una sensibilidad del 85,4% y una especificidad del 95,2%, el test Wako (Wako Pure Chemical Industries, Japón), estudiado por Fujita y colaboradores (Fujita, Takamura, Nagahara, & Hashimoto, 2006), cuyos parámetros diagnósticos fueron S=95% y E=84%, el test B-G Star (Maruha Corporation, Japón) que se utilizan en Japón, y finalmente Fungitell (Associates of Cape Cod Inc., EEUU) cuya utilización está autorizada en Estados Unidos y Europa mostrando una capacidad diagnóstica con una sensibilidad del 64,4% y una especificidad del 92,4% (Pontón, 2009). Martínez-Jiménez y colaboradores observaron unos valores de S=83,9%, E=91,8%, VVP=86,7% y VPN=90% (Martínez-Jiménez et al., 2015).

Donato y colaboradores obtuvieron una sensibilidad y especificidad del 60% y 92%, respectivamente. El valor predictivo positivo fue del 60% y el valor predictivo negativo del 92% en un grupo de pacientes ingresados en la unidad de cuidados intensivos (Donato et al., 2017). Por otro lado, Levesque y colaboradores en un estudio de 27 pacientes que han sufrido un trasplante de hígado, la sensibilidad, especificidad, valor predictivo positivo y valor predictivo negativo fueron respectivamente del 75%, 65%, 17% y 96% (Levesque et al., 2017). Cabe destacar que en la mayoría de las publicaciones el alto valor VPN obtenido en las pruebas de diagnóstico, hace del β -glucano un buen marcador para el diagnóstico de la CI.



1.7.2.2 Detección de D-arabinitol

El D-arabinitol es un metabolito producido por la mayoría de las especies patógenas de *Candida* (*C. albicans*, *C. tropicalis*, *C. parapsilosis*) pero no por *C. glabrata* o *C. krusei* (Bernard, Christiansen, Tsang, Kiehn, & Armstrong, 1981; Hsu et al., 2011; Tumbarello et al., 2007).

Ya en 1979, Kiehn y colaboradores estudiaron mediante cromatografía de gas-líquido la presencia de este metabolito en aquellos individuos con CI (Kiehn, Bernard, Gold, & Armstrong, 1979).

La detección de este metabolito se basa en su reacción con la D-arabinitol deshidrogenasa, que produce D-ribulosa y NADH; este último producto es el que se detecta por espectrofluorimetría o bien enzimáticamente o mediante cromatografía de gases (Arendrup et al., 2010; Christensson, Sigmundsdottir, & Larsson, 1999; Hui et al., 2004; Pérez-García et al., 2017; Sigmundsdottir, Larsson, Wiebe, Björklund, & Christensson, 2007). Yeo y colaboradores han desarrollado una técnica de diagnóstico del D-arabinitol basándose en la detección por espectrofluorimetría a partir de una D-arabinitol deshidrogenasa de *C. albicans*. Es un método rápido (3,5 minutos por ensayo) y preciso con resultados muy prometedores. Se observaron una media de D-arabinitol/creatinina 2,74 y 2,23 $\mu\text{M}/\text{mg}/\text{dl}$, respectivamente, en comparación con 1,14 y 1,23 $\mu\text{M}/\text{mg}/\text{dl}$ en pacientes del grupo control (Yeo, Zhang, Schafer, Campbell, & Wong, 2000).

Sin embargo, este método resulta laborioso y utiliza una metodología compleja que no está disponible en el ámbito hospitalario, limitándose a unos pocos centros.

Otro inconveniente relacionado con este marcador es que puede ser liberado también por organismos colonizadores, dificultando por lo tanto la discriminación entre una CI y una colonización por *Candida*; también se ha observado su presencia en suero y en orina humana debido a cambios en la propia microbiota, a tratamientos con antibióticos o quimioterápicos, a la administración de esteroides y corticosteroides, o durante insuficiencia renal (Jones, 1990).

Debido a ello, se sugirió la normalización de los niveles de D-arabinitol mediante su expresión como relación D-arabinitol/creatinina (Wong, Bernard, Gold, Fong, &



Armstrong, 1982) o D-arabinitol/L-arabinitol (Larsson, Pehrson, Wiebe, & Christensson, 1994), compensando de esta forma su presencia debido a otras causas.

1.7.2.2.3 ADN

La detección de ADN, aprovecha la tecnología de la PCR (reacción a cadena de la polimerasa) a tiempo real, que es uno de los mayores avances que hoy en día se han producido en este campo (Pontón & del Palacio, 2007). Con respecto a los métodos convencionales, las principales ventajas que ofrece esta técnica es el menor tiempo necesario para la obtención de resultados, una mayor sensibilidad y la posibilidad de identificación a nivel de especie. Sin embargo, uno de los aspectos de la técnica a los que hay que hacer frente es la falta de estandarización, lo que hace difícil su aplicación en la clínica para el diagnóstico de las candidiasis, ya que cada grupo utiliza metodologías desarrolladas en su propio laboratorio (Quindós, Eraso, López-Soria, & Ezpeleta, 2012). Existen algunas pruebas comercializadas para el diagnóstico de la CI, entre ellas cabe destacar LightCycler®*SeptiFast* (Roche Molecular System, EEUU) y la plataforma TaqMan system (Perkin Elmer, Applied Biosystem, EEUU). Sin embargo, todavía no existe consenso sobre cuál es la mejor muestra (sangre completa, suero o plasma) a utilizar para la extracción de ADN. Más recientemente se ha desarrollado una técnica de diagnóstico que se basa en la combinación de la resonancia magnética con nanopartículas para la detección e identificación de todas las especies de *Candida* (Mylonakis, Zacharioudakis, Clancy, Nguyen, & Pappas, 2018). Es una técnica muy rápida que permite la detección de *Candida* a partir directamente de una muestra de sangre. T2 Biosystem realiza una amplificación de ADN a partir de una muestra de sangre completa y mediante resonancia nuclear detecta amplicones específicos con un límite de detección hasta 1 CFU/ml entre 3 y 5 horas (T2 Biosystems, Inc.; EEUU). La técnica ha demostrado una especificidad general del 99,4%; siendo del 98,9% para *C. albicans*/*C. tropicalis*, del 99,3% para *C. parapsilosis* y del 91,1% para *C. krusei*/*C. glabrata*. En cambio, la sensibilidad fue del 92,3% para *C. albicans*/*C. tropicalis*, 94,2% para *C. parapsilosis*, y del 88,1% para *C. krusei*/*C. glabrata*. El valor predictivo negativo varió entre 99,5 y 99% (Mylonakis et al., 2015).



1.8 The immune system

C. albicans is part of the normal human microbiota, and is associated to the mucosa surfaces of the oral cavity, gastrointestinal tract and vagina. Although it exists as a commensal under normal conditions, *Candida* can switch to a pathogenic organism able to infect various tissues and cause systemic disease (Romani, 2008). The host has three main mechanisms of defense against *C. albicans*: a physical barrier, the innate immune system and the adaptive immune system (Janeway Jr & Medzhitov, 2002).

The physical barrier, such as skin and mucous membranes, has the natural ability to separate the host from the external environment. However, when fungi break this barrier, a series of innate mechanisms come into play mediated by cells, cellular receptors and humoral factors.

1.8.1 Innate immunity

The innate immunity has the capacity to distinguish self from non-self. It lacks of certain specificity and activates adaptive immune system by specific signals (Janeway Jr & Medzhitov, 2002; Romani, 2004). It includes several kinds of cells: phagocytes such as neutrophils, monocytes and macrophages; dendritic cells (DC), natural killer cells (NK), T lymphocytes and non-hematopoietic cells (Blanco & Garcia, 2008; Romani, 2002). On the one hand, it has the ability to attack directly fungal pathogens through phagocytic processes or through the secretion of microbicidal compounds. On the other hand, it presents a constitutive role to instruct cells of the adaptive immunity through the production of pro-inflammatory cytokines and the induction of co-stimulatory activity by antigen presentation cells and phagocytosis (Romani, 2004). More in general, the majority of innate mechanisms are activated when invariant patterns of the pathogens, also called pathogens-associated patterns (PAMPs), are recognized by a set of pattern recognition receptors (PRRs), Toll-like receptors (TLRs) and C-type lectins of the host (Figueiredo, Carneiro, & Bozza, 2011).



1.8.2 Adaptive immunity

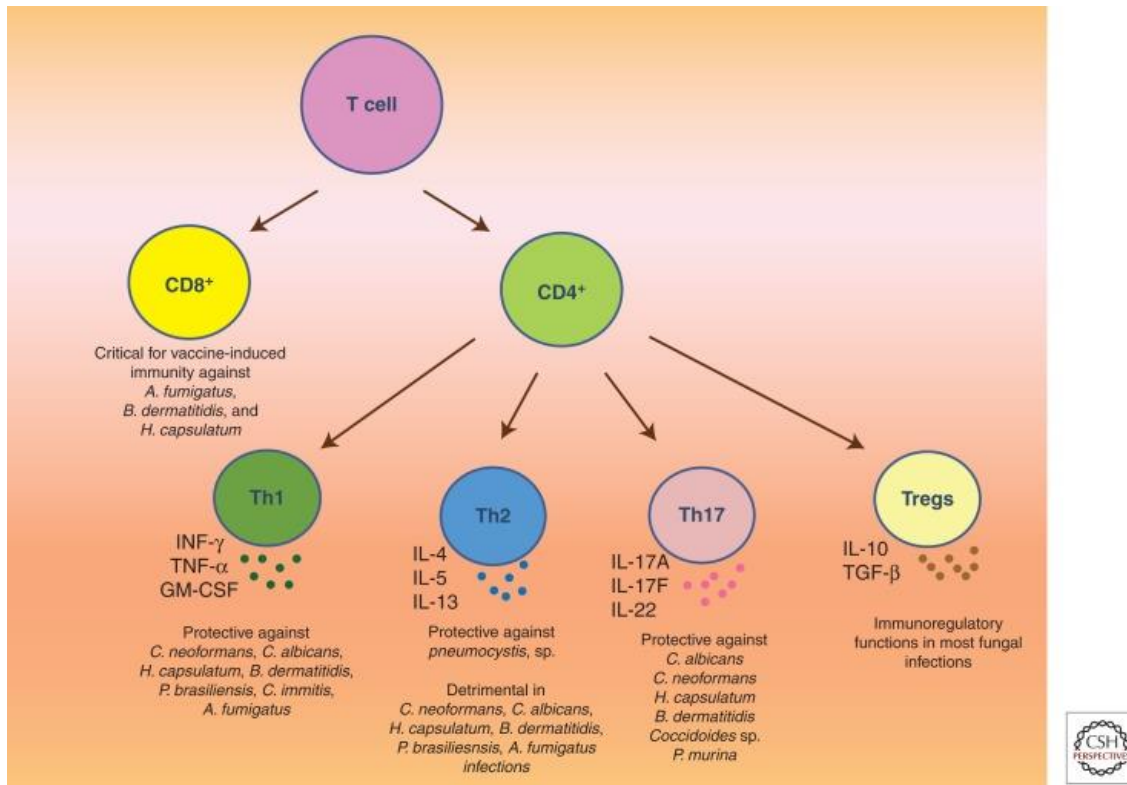
The adaptive immunity takes place in secondary lymphoid organs that are spleen, lymph nodes and MALT (Mucosa-associated lymphoid tissue). It is mainly mediated by lymphocytes and macrophages. It is more specific, clonal and develops memory after an initial encounter with pathogens (Medzhitov & Janeway, 1997).

There are two big groups of lymphocytes known as B and T; the last one can be distinguished in T-helper that show CD4 molecules on their surface, and T-cytotoxic (Tc) or T-regulators (Treg) that show CD8 molecules on the surface. T-helper lymphocytes can be categorized in 5 groups: Th1, Th2, Th9, Th17 and T_{Reg} cells (Jiang, 2016).

More in general the adaptive response starts when antigen-presenting cells (APC) which express major histocompatibility complex molecules (MHC) on their surface, present the antigens to naïve T lymphocytes (Th0) that recognize these antigens through their receptor (TCR). Depending on the type of pathogen, Tc-lymphocytes are able to recognize antigens through MHC class I or II (Regueiro, López-Larrea, González-Rodriquez, & Martinez-Nave, 2011).

One of the most important APC cells are DCs that lie at the intersection of innate and adaptive immunity. DC cells secrete cytokines and express specific co-stimulatory molecules helping the differentiation of naïve T-cell into a T-helper (Th) phenotype (Verma, Wuthrich, Deepe, & Klein, 2014).

Cytokines are small proteins secreted by specific cells that carry signals locally between cells. Depending on the secreted cytokine by the APC, the transition of Th0 could be directed to Th1, Th2 Th17 or Treg (Figura/Figure 1.5).



Figura/Figure 1.5 Schematic illustration of different T lymphocytes and their roles in combating different fungal pathogens. Adapted from (Verma et al., 2014).

DCs can activate Th1 cells through TLR signaling, which in turn help to optimize the activation of phagocytes at the site of infection (Lin et al., 2009). The release of interleukin-12 (IL-12) by APC is critical for the Th1 role. These cells response to the fungal infection releasing pro-inflammatory cytokines such as IFN- γ (interferon- γ), TNF- α (tumor necrosis factor alpha) and GM-CSF (granulocyte-macrophage colony-stimulating factor) (Romani, Puccetti, & Bistoni, 1997; Verma et al., 2014). IFN- γ manifests pleiotropic effect on immune cells; it induces classical activation of macrophages, critical to kill intracellular fungal pathogens (Verma et al., 2014).

On the contrary, Th2 response is stimulated by IL-4 and IL-13. Th2 cells liberate IL-4, IL-5, IL-6 and IL-10, activating B-cell clones. These cells are involved in humoral response against fungal allergy and immune protection against extracellular microorganisms (Cenci et al., 1999). IL-5 can limit the Th1 response as well as activating M2 macrophages, harmful to those patients with severe fungal infections (Jiang, 2016). By the way, cytokines secreted by Th1 lymphocytes inhibit Th2 cells and vice versa.



The secretion of IL-17A, IL-17F and IL-22 promotes Th17 cells. In addition, TGF- β (transforming growth factor beta) and IL-6 are required for the initial differentiation of Th-17, and IL-23 is necessary to maintain and expand these cells (Verma et al., 2014; Zúñiga, Jain, Haines, & Cua, 2013). Defective Th17 cells render patients susceptible to mucosal fungal infections (Jiang, 2016).

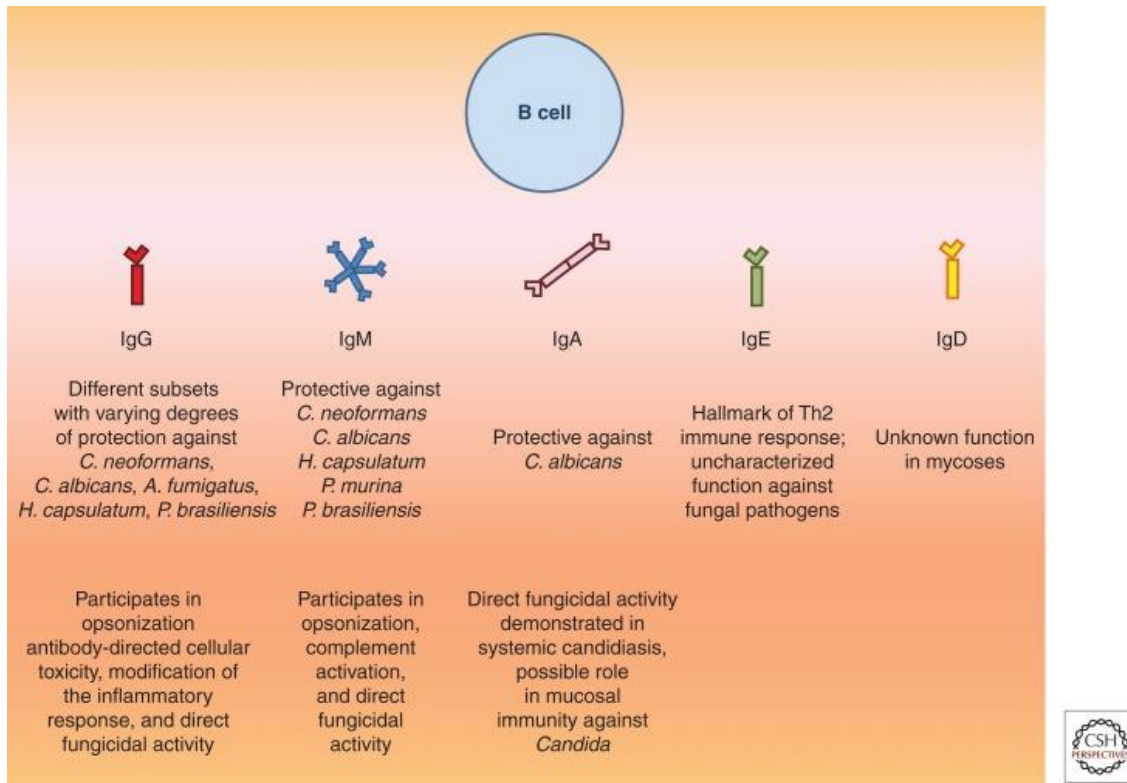
On the other hand, Treg lymphocytes contribute to regulate pro-inflammatory immune response generated against infectious agents to limit collateral damage to the host. This complicated system includes secretion of inhibitory cytokines IL-10, TGF- β , or IL-35, and repression of IL-2 release (Goodman, Cooper, & McCormick, 2012; Verma et al., 2014). Despite their immunosuppressive function, Treg cells have a positive impact by promoting durable antifungal immunity (Verma et al., 2014).

Finally CD8+ T cells, specific against viral pathogens and tumors (Verma et al., 2014), can also block fungal growth such as *C. albicans in vitro* (Jiang, 2016). They release IFN- γ and IL-17 as a memory mechanism to coordinate the resolution of the pathogens (Verma et al., 2014).

Meanwhile, B-cells are the main components of the humoral immunity with the objective to eliminate extracellular pathogens and avoid their dissemination. In order to turn activated, naïve B-cell need signals from Th cells, cytokine secretions or membrane molecules that link to B-cells receptors.

B-mature cells derive from a particular cell. Therefore, the antibodies and their differentiated progenies are able to recognize the same epitope of a specific antigen. Only a small minority survive as memory cells, able to recognize the same epitope and protect the host from future infection caused by the same pathogens (Regueiro et al., 2011).

The antibodies or immunoglobulins (Igs) are glycoproteins produced by B-cells. All of them are formed by four polypeptide chains, two heavy (H) and two light (L), linked by disulfide bound. Depending on their heavy chain class (α , δ , ϵ , γ and μ), there are five classes of antibodies (IgA, IgD, IgE, IgG and IgM) that play different functions in fungal disease (Figura/Figure 1.6).



Figura/Figure 1.6 Schematic illustration of immunoglobulins and their roles in combating different fungal pathogens. Adapted from (Verma et al., 2014).

Antibodies are located in the serum and in tissue fluids, where they can be found in solution or bound to the B-cell membrane as a specific part of B-cell receptor (BCR). They can be used for basic research applications due to their high specificity.

The antibodies can mediate protection by direct and indirect mechanisms. Direct protection results in inhibition of growth or microbicidal activity when Igs bind to the pathogens (Verma et al., 2014), or when they bind to the external surface of the fungus and prompt the alteration of gene expression and fungal metabolism (Brena et al., 2011; McClelland, Nicola, Prados-Rosales, & Casadevall, 2010). Indirect activities relate to Ig-mediated resolution of the infection by improvement of microbicide potential of effector cells (Verma et al., 2014). Other well-known mechanisms are opsonization, activation of the complement pathway and antibody-directed cell toxicity (ADCC).

1.8.3 Antifungal immunotherapy

The increasing number of *C. albicans* infection cases has prioritized the development of alternative treatments (Ibrahim et al., 2005). One of the main



impediments to fungal vaccine advance is that patients most susceptible to fungal infections are those with a weak immune response (Segal et al., 2006).

1.8.3.1 Enhancement of immune activity

Nowadays there exist different approaches to fight fungal infection that include the application of effectors and regulatory cells (granulocytes, antigen-specific T cells) or the administration of recombinant cytokines and growth factors (IFN- γ), granulocytes and granulocytes-macrophages colony stimulating factors (G-CSF, GM-CSF), TNF- α or IL-15, and vaccinations (Posch, Steger, Wilflingseder, & Lass-Flörl, 2017).

In infections caused by *C. albicans*, *in vitro* TNF- α proved to be a potent activator of the fungicidal activity of PMN (polymorphonuclear leucocyte) cells (Djeu, Blanchard, Halkias, & Friedman, 1986; Ferrante, 1989; Roilides, Lamaignere, & Farmaki, 2002). In this regard, in a murine model of IC, PMN cells showed an increased intracellular killing activity of *C. albicans* in combination with fluconazole and T α 1 (thymosin- α 1) (di Francesco et al., 1994). T α 1, a synthetic version of a thymic derived biological response modifier, was the first thymosin in clinical use. It is approved in over than 35 countries for the treatment of hepatitis B and C, and as an immune stimulant and adjuvant (Goldstein & Goldstein, 2009; Posch et al., 2017).

Besides, macrophage colony stimulating factor (M-CSF) enhances monocytes and macrophage functions, such as phagocytosis, cytokine production, chemotaxis and superoxide production (Nemunaitis, 1998). Cenci et al. observed that mice infected with *C. albicans* had higher survival rate after prophylactic administration of M-CSF (Cenci et al., 1991). In addition, survival rate of rats treated with M-CSF in combination with fluconazole also increased (Nemunaitis, 1998; Posch et al., 2017). Other studies evaluating the usage of GM-CSF alone or in combination with IFN- γ , observed an improved fungicidal activity of innate phagocyte cells (Roilides, Blake, Holmes, Pizzo, & Walsh, 1996; Safdar et al., 2013) in humans (Safdar et al., 2013) and in animal models (Roilides et al., 1996). In addition, several studies have focused on a combinatorial therapy based on antifungal drugs and therapy increasing cytotoxicity of APCs in patients with *Candida* and *Aspergillus* infections. In this regard, IFN- γ was responsible for the secretion of pro-inflammatory cytokines



(IL-1 β and TNF- α) and up-regulation of MHCII receptor of leukocytes, promoting a higher induction of T cell response (Delsing et al., 2014).

Some immunotherapy studies have characterized the protective role of monoclonal antibodies in *C. albicans* infections. The efficacy depends on the isotypes, the titer of fungal specific antibodies, the presence of protective and non-protective antibodies, and other host factors (Maitta et al., 2004; Rivera & Casadevall, 2005). This kind of treatment is especially indicated to those individuals suffering from some immunological disorder and therefore they are unable to produce an adequate immunological response (Posch et al., 2017). The mAb 2G8, directed against the β -glucan containing laminarin, inhibited the growth of *C. albicans in vitro* and protected against this fungus infection (Torosantucci et al., 2009). Another monoclonal antibody, called Mycograb® (scFv anti-HSP90), reduced mortality of *C. albicans* infection in a clinical trial improving fungal clearance (Pachl et al., 2006), however the production of Mycograb (efungumab) was discontinued in 2010 (Novartis).

In the same way, resulting extremely necessary enhances humoral immunity against invasive fungal infections by application of antibodies or vaccinations.

In the last few years, several studies confirmed the immunogenicity and the efficacy of different vaccines against *Candida* in an animal model of infection (Cassone & Casadevall, 2012; Ibrahim et al., 2013; Ibrahim, Spellberg, Avanesian, Fu, & Edwards, 2006; Spellberg et al., 2006). Polysaccharide components of the fungal cell wall, proteins and attenuated fungal strains have been investigated as possible vaccines (Wang et al., 2015).

The interaction between *Candida* and the host is complex and we still do not totally understand the motifs that bring the fungus to become a pathogen from saprophytic organism.

Increasing the humoral response through the administration of fungal components could be a possible approach to trigger the host immune activity. In this regard, vaccines work by eliciting an immune response and subsequent immunological memory that mediates protection from an infection or a disease (Iannitti, Carvalho, & Romani, 2012).



The β -MN (β -mannan) of the cell wall phosphomannan of *C. albicans* is an attractive component for a vaccine. Anti-mannan antibodies can be detected in most human sera and some of them are protective against IC (Moragues et al., 2014). Since polysaccharides by themselves do not have immunostimulatory activity (Ranta et al., 2012), they require the attachment to protein carriers. A synthetic glycopeptide vaccine, called β -(Man)₃-Fba, based on mannan component that contains the N-terminal portion of the *C. albicans* protein fructose-bisphosphate aldolase (Fba) as the glycan carrier, and coupled to tetanus toxoid to improve immunogenicity, has been used as vaccine in a murine model of infection. Longer survival rates and lower fungal burden in kidneys were associated with the protection mediated by specific IgG and IgM antibodies and by the induction of T cell-dependent immune memory (Xin et al., 2012).

Similarly, laminarin, a β -glucan from the brown alga *Laminaria digitata*, has been conjugated with the diphtheria toxoid CRM197 (a carrier protein). This conjugate showed immunogenic and protective activity in a murine experimental model of candidiasis (Torosantucci et al., 2009).

In addition, the immune system has a chitin recognition mechanism and this compound is able to stimulate macrophages conferring protection against IC. Chitin potentiates the Th1 response through cytokines IL-12, INF- γ and TNF- α production and a decrease of IL-4 (Gorzelanny, Pöppelmann, Pappelbaum, Moerschbacher, & Schneider, 2010; Ozdemir et al., 2006; Rementería et al., 1997).

There are different studies regarding the Als3. Ibrahim and colleagues studied several vaccine against the IC including rAls1p-N (recombinant N-terminus of Als1p) (Ibrahim et al., 2005; Ibrahim et al., 2006; Spellberg et al., 2005), rAls3p-N (recombinant N-terminus of Als3p) (Lin et al., 2009; Spellberg et al., 2008) and NDV-3 (recombinant N-terminus of Als3p formulated with alum adjuvant) (Ibrahim et al., 2013; Schmidt et al., 2012). NDV-3 has been tested in a Phase I clinical trial, and has shown to be well tolerated even at 300 mg (Schmidt et al., 2012). Now, it is in phase 1b/2a clinical trial again in patients diagnosed with VVC (Sampaio & Pais, 2017). All of them resulted effective in a murine model of IC (Wang et al., 2015). The family of secreted aspartyl proteinases (SAP) has been related with protection against vulvovaginal candidiasis (VVC) (De Bernardis et al., 2012). There is a vaccine



based on Sap2p (PEV7; Pevion Biotech AG, Switzerland), currently in phase I clinical trial, which generates protection against VVC caused by *C. albicans* (Edwards Jr, 2012; Wang et al., 2015). The protein Hsp90, a stress-induced cellular chaperone, is present in the cell wall of *C. albicans* yeast and hyphae (Moragues et al., 2014). Immunization with a recombinant Hsp90 has revealed Th1 and Th2 responses in an animal model of IC (Mašek et al., 2011). The production of Mycograb, a monoclonal antibody directed against Hsp90, was discontinued in 2010 due to quality aspects and safety concerns related to the treatment of patients with invasive candidiasis.

Hyr1p is a glycosyl phosphatidyl inositol (GPI)-anchored mannoprotein expressed on the cell wall and related to the hyphal growth of *C. albicans* (Bailey et al., 1996; Cassone, Bromuro, Chiani, & Torosantucci, 2010). It mediates resistance to phagocytic killing *in vitro*, and vaccination of immunocompetent mice with the recombinant N-terminal fraction of this protein (rHyr1p-N) improved the survival rate of an animal model of IC (Luo et al., 2010).

The combination of different antigens in a vaccine could benefit of a better protection based on a broader immune response and protective antibodies, reducing the incidence of fungal immune evasion (Wang et al., 2015). The proposal of Cassone and colleagues (Cassone, 2013) with a combined vaccine of Als3-N and Sap2p is currently on clinical trial. Most experiments on fungal vaccines have been performed in animal models. The implementation to the human being still causes concern related to efficacy, toxicity, standardization of the fabrication process, administration, costs and clinical trials still have a long way to run.

Despite all these shortcomings, immunotherapy could be an alternative route of treatment for fighting microbial diseases lacking other adequate therapeutic options.

1.9 Interaction between *Candida albicans* and the epithelium and pathogenicity

The increasing prevalence of *C. albicans* infections supports the case for understanding the interactions between *Candida* and host epithelium, including the pathogenic aspects of the fungus.



The role of epithelial cells during the infection of *Candida* has been addressed recently, as long as it is the first-line defense barrier and is continuously exposed to different external agents. As a result, epithelial cells play a critical role in commensal/pathogen discrimination, immunity and damage repair (Moyes, Richardson, & Naglik, 2015; Moyes & Naglik, 2011; Naglik, Moyes, Wächtler, & Hube, 2011; Naglik, Tang, & Moyes, 2013).

1.9.1 Adhesion

Epithelial cells play a crucial role in the prevention of fungal invasion and intervene in the initial stages of fungal contact, colonization and invasion. The adhesion of *C. albicans* to the epithelial cells is one of the most important steps for the colonization and growth of the fungus. This complex process involves multiple factors including *Candida* adhesins present in the surface of the cells wall and host ligands in the surface of the epithelial cells. The adhesins vary depending on the morphological status of the fungus (Chaffin, 2008) and interact with the receptors of the epithelial cells (Tabla/Table 1.2).



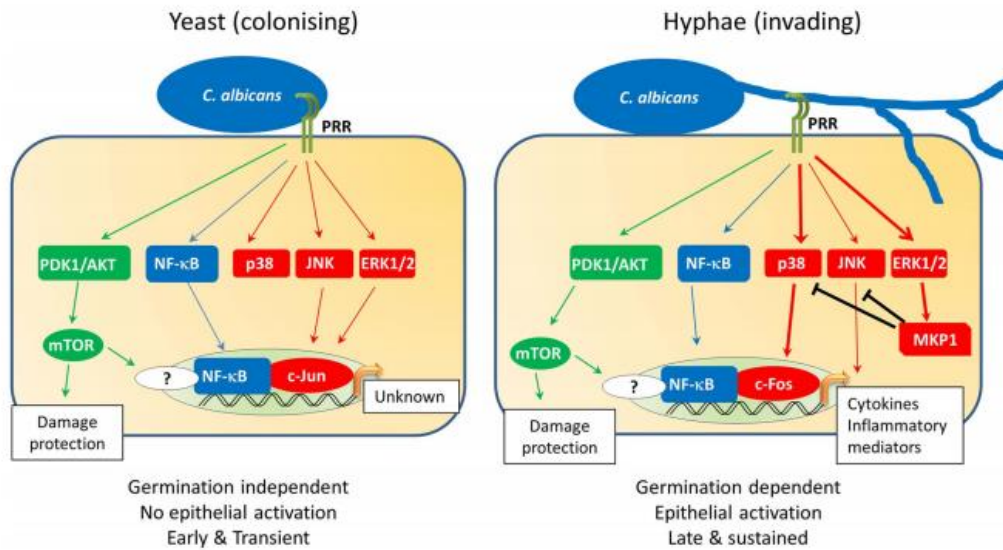
Table/Table 1.2 *C. albicans* adhesins that mediate the binding between the epithelial cells surface and their host target molecules. Adapted from (Moyes et al., 2015).

Candida adhesin	Cellular receptor/substrate	Reference
Eap1p	Polystyrene, epithelial cells	(Li & Palecek, 2008; Li & Palecek, 2003)
Iff4p	Plastic, epithelial cells	(Fu, Luo, Spellberg, Edwards, & Ibrahim, 2008; Kempf et al., 2007)
Hwp1p	Host cell transglutaminase substrates	(Staab et al., 2004; Sundstrom, 2002; Sundstrom, Balish, & Allen, 2002)
Int1p	iC3b	(Hostetter, 1994)
Als3p	E-Cadherin, EGFR/Her2	(Phan et al., 2007; Zhu et al., 2012)
GlcNAc-binding protein	N-Acetylglucosamine	(Critchley & Douglas, 1987a; Critchley & Douglas, 1987b)
Fimbrial adhesin	β GalNAc(1-4 β -Gal)	(Yu et al., 1994)

1.9.2 Recognition and activation

Once *C. albicans* cells have adhered to the surface of epithelial cells, they are recognized. This event is extremely important because it allows discriminating between the commensal and pathogenic phases of the fungus.

The epithelial cells respond to the presence of the fungus activating five key intracellular signaling pathways the 3 main Mitogen Activated Protein Kinase (MAPK) pathways (JNK, p38 and ERK1/2), the Phosphatidylinositide-3-kinase (PI3K) pathway and the Nuclear Factor-kappa-enhancer of B cell function (NF-kB) pathway (Figura/Figure 1.7) (Naglik, Richardson, & Moyes, 2014).



Figura/Figure 1.7 *C. albicans* induces a biphasic innate immune MAPK response discriminating between the yeast and hyphal forms of *C. albicans* in epithelial cells (Naglik et al., 2014).

The MAPK pathways show a more dynamic activation profile in comparison with NF-κB and PI3K pathways (Moyes et al., 2010; Moyes et al., 2015; Moyes et al., 2011; Naglik et al., 2014)

The MAPK signaling has 2 phases. The initial phase is transitory and morphology-independent that appears during the first five minutes of the infection and rapidly disappears by 1 hour. In this phase, there is an activation of c-Jun transcription factor dependent of JNK and ERK1/2.

Two hours after the infection, a sustained reactivation of all 3 MAPK pathways follows, but in this case with the activation of c-Fos transcription factor (driven by p38) and the phosphorylation of the MAPK phosphatase, MKP1 (driven by ERK1/2). This second phase is morphology-dependent, considering that only hyphae producing strains that are able to induce this response (Moyes et al., 2010; Moyes et al., 2012; Moyes et al., 2015) and it is affected by the fungal burden (Moyes et al., 2010).

The second epithelial signaling response induced by hyphae (c-Fos), but not the yeast-induced one (c-Jun), is fundamental for the production of most pro-inflammatory cytokines secreted by epithelial cells in response to *C. albicans*, such as G-CSF, GM-CSF, IL-1 α , IL-1 β and IL-6 (Moyes et al., 2015).



1.9.3 Invasion, induced endocytosis and active penetration

Adhesion and recognition of *C. albicans* by epithelial cells is leading to the invasion of the fungus. One of the earliest effect of this interaction is the cytoskeletal reorganization within the host cell, which occurs within the first 4 hours of initial contact. The adherent hyphae induce host endocytosis mechanisms involving actin and other proteins associated with clathrin-mediated endocytosis (Moreno-Ruiz et al., 2009; Moyes et al., 2015; Villar & Zhao, 2010; Zhu et al., 2012). This is a passive host-driven process because dead *C. albicans* hyphae are also endocytosed (Park et al., 2005).

Several fungal proteins have been identified as promoters of the induced endocytosis. In this regard, the adhesin/invasins Als3p and Ssa1p (heat shock protein 1) interact with the epithelium cells surface through E-cadherin (Phan et al., 2007; Sun et al., 2010), RTKs (receptor tyrosin kinases), Her2 (Glutamyl-tRNA(Gln) amidotransferase subunit A) and EGFR (epidermal growth factor receptor) (Zhu et al., 2012). As a result, the phosphorylation of EGFR and Her2 (the mechanisms yet unidentified) leads to induced endocytosis of *C. albicans* through clathrin-mediated phenomena (Moreno-Ruiz et al., 2009). In addition to Als3p and Ssa1p, Rim101p has been proposed to play a role in induced endocytosis, since mutants of this pH regulation transcription factor (*rim101Δ/Δ*) present attenuated capacity to promote endocytosis by oral epithelial cells (Nobile et al., 2008a).

On the other hand, mucosal surfaces such as oral cavity and vagina lumen that consist of stratified, squamous epithelium are unlikely to support this active endocytic process to internalize fungal cells. Since these kinds of surfaces are terminally differentiated and non-proliferative, they need alternative methods to internalize *C. albicans*, which are referred to as active penetration (Moyes et al., 2015). Contrarily to the endocytic mechanism, active penetration requires viable hyphae in order to invade the epithelial cells or penetrate between them (Dalle et al., 2010; Wächtler, Wilson, Haedicke, Dalle, & Hube, 2011). Although the entire mechanism and the proteins involved are not totally understood, some adhesion proteins such as Als3p, relevant for the endocytic mechanism, does not play an



essential role in active penetration (Wächtler et al., 2011). In reference to the secreted fungal enzymes such as lipases and phospholipases or proteases, none of them has been related with active penetration except the secreted aspartic protease family (SAP). Nevertheless, this role seems controversial because some studies have reported that Sap proteins damage the oral epithelium (Naglik et al., 2008) and vaginal mucosa (Villar, Kashleva, Nobile, Mitchell, & Dongari-Bagtzoglou, 2007), while other authors referred that *sap* deleted mutant strains conserved their ability to damage these epithelia (Lermann & Morschhäuser, 2008).

1.9.4 Damage

The damage is the final step during the infection process. It is a combination of two mechanisms, necrosis and apoptosis. Recently, a new damage mechanism called pyroptosis has been identified in macrophages caused by *C. albicans*, however, it is currently unknown if this inflammasome-driven mechanism system is activated during epithelial cells damage (Moyes et al., 2010; Moyes et al., 2012; Moyes & Naglik, 2011).

Necrosis is characterized by swelling of mitochondria and an increment of plasma membrane permeability (Naglik et al., 2011). Meanwhile apoptosis involves a series of events usually triggered by a family of cysteine aspartic proteases called caspase, that cause biochemical breakdown of cellular contents, resulting in cell death.

An *in vivo* study showed that damage caused by hyphae was higher than the damage promoted by non-hyphal mutants. Nevertheless, some mutants that still produced hyphae were able to induce reduced damage, suggesting that specific hyphae effectors and not only mycelia are necessary to induce damage (Peters et al., 2014). In the same way, several studies blocking the endocytosis process or using mutants with reduced epithelial endocytosis, reported reduced damage (Martinez-Lopez, Park, Myers, Gil, & Filler, 2006; Nobile et al., 2008a; Park et al., 2005; Phan et al., 2007). In order to cause damage, the endocytosis process is not enough, but the *C. albicans* cells endocytised have to be viable and bear genes expressing damage factors (Dalle et al., 2010; Moyes et al., 2010; Nobile et al., 2008a). Similarly, invasion alone is not enough to generate damage of the cells; this finding is supported by experiments with *C. albicans eed1Δ/Δ* (Epithelial Escape and Dissemination1)



mutants that can adhere and invade the cells, but are not able to cause damage (Zakikhany et al., 2007).

A large panel of genes related with the damage process, including adhesion or invasion, has been studied (Wächtler et al., 2011). The results showed that genes involved with all three of these functions play an important role in the yeast-hyphae morphological switch. These results give more relevance to the morphological switch during *C. albicans* infection.

1.9.5 Mucosal immunity against *Candida albicans*

The recognition of *C. albicans* by epithelial cells brings to the activation of a cytokine pro-inflammatory response profile (Dongari-Bagtzoglou & Fidel Jr, 2005; Moyes et al., 2010; Villar, Kashleva, Mitchell, & Dongari-Bagtzoglou, 2005; Weindl et al., 2007), including IL1 α/β , IL-6, G-CSF, GM-CSF and TNF α , as well as the chemokines RANTES, IL-8 and CCL20.

Up to now, the direct effect of these cytokines on epithelial protection remains unclear; however, they are involved in the recruitment and activation of lymphoid and myeloid cells in the mucosal layer (Weindl et al., 2007).

At the same time, *C. albicans* infection promote an increase of the expression of matrix metalloproteases (MMPs), being responsible for the epithelium remodeling and modulation of the barrier function (Claveau, Mostefaoui, & Rouabhia, 2004).

The recruitment of immune cells caused by cytokine secretion includes neutrophils, dendritic cells and T cells. Neutrophils can induce protection of epithelial cells mediated by the upregulation of TLR4 (toll-like receptor) (Weindl et al., 2007). At the same time, they are able to kill directly *C. albicans* cells through ingestion, killing and degranulation, or mediated by the recently discovered Neutrophils Extracellular Traps (NETs) system (Moyes & Naglik, 2011). Similarly, IL-8 recruits neutrophils and induces a Th17 response mediated by the secretion of CCL20 (Ghannam et al., 2011).

Several studies with mice deficient in IL-17 and infected with *C. albicans* showed an increased susceptibility to oropharyngeal candidiasis, dermal candidiasis (Conti et al., 2009) and systemic candidiasis (Huang, Na, Fidel, & Schwarzenberger, 2004;



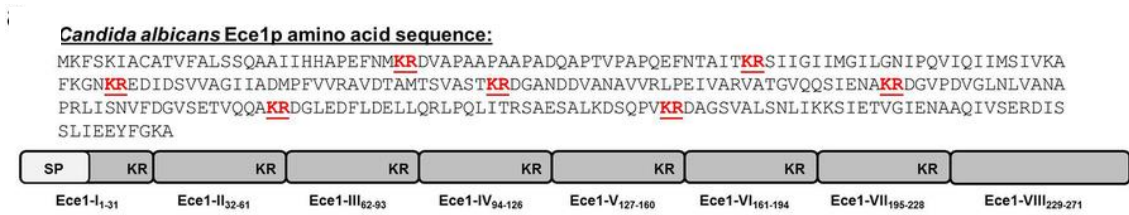
Saijo et al., 2010; van de Veerdonk et al., 2010). The role of Th17 related to *Candida* immunity is quite controversial, there was evidence of an increased yeast burden in a mice model of infection with a heightened IL-23/Th17 response (Zelante et al., 2007).

IL-22 and IL-17 have been associated to the anti-*C. albicans* immunity. IL-17 acts on epithelial cells and neutrophils mediating a relation between the innate and adaptive immune response (Moyes & Naglik, 2011). Meanwhile IL-22, in addition to presenting similar effects to IL-17 on epithelial cells, has been suggested to control the growth of *Candida* yeasts and the integrity of the epithelial layer during the infection (De Luca et al., 2010).

1.9.5.1 Ece1p (extent of cell elongation 1)

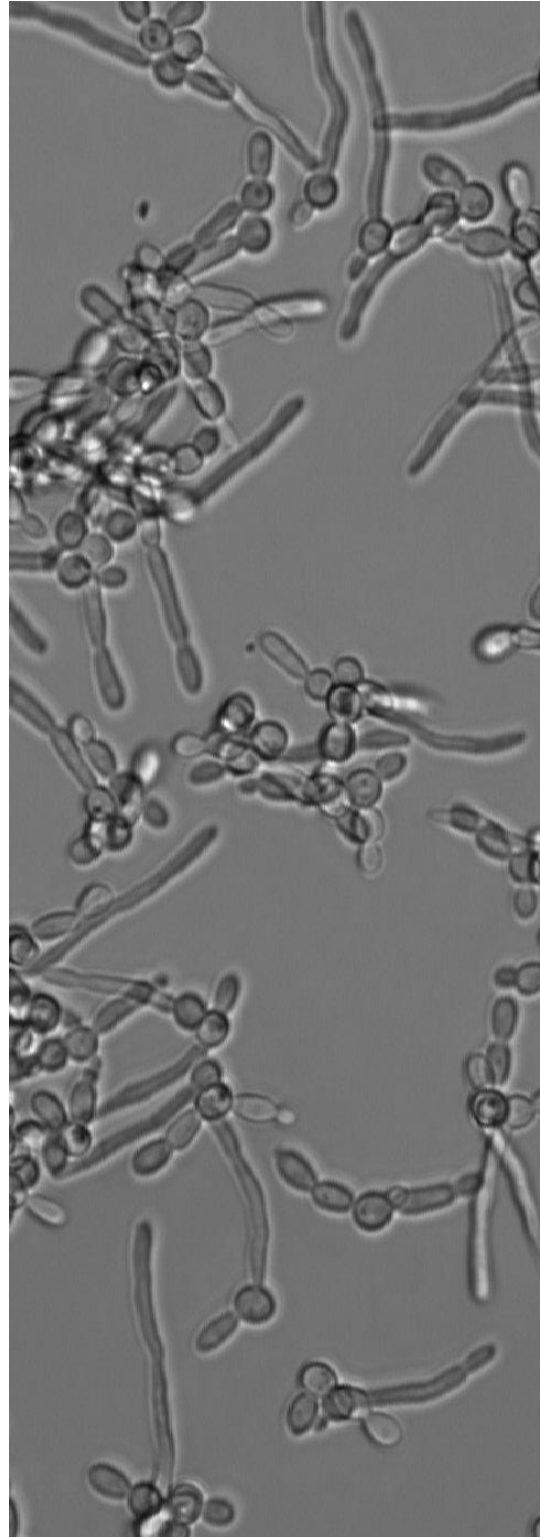
Ece1p is a specific protein of the mycelial phase of *C. albicans* that contains 271 amino acids and was described by Birsie and colleagues (Birse, Irwin, Fonzi, & Sypherd, 1993). Although it is upregulated in association with the morphological change, it is not essential to hyphae formation (Birse et al., 1993; Moyes et al., 2016). Over 100 mutant strains of *C. albicans* were studied in order to identify the fungal factor responsible for driving the p-MKP1/c-Fos/cytokine secretion in oral epithelial cells (Moyes et al., 2010; Moyes et al., 2016). Only the *ece1Δ/Δ* mutant was unable to activate this response or damage epithelia, although it was able to form hyphae and penetrate the epithelium. Therefore, Ece1p was implicated in the development of innate immune response and damage production to the epithelial cells (Moyes et al., 2016).

Ece1p is the substrate of Kex2p, a Golgi-located protease that cleaves the protein after KR (lysine-arginine) motifs yielding 8 fragments named Ece1-I to Ece1-VIII (Figura/Figure 1.8). Moyes and colleagues observed that peptide Ece1-III_{62-93KR} was the active region of Ece1p responsible for damage induction and cytokine production by epithelial cells. This peptide named Candidalysin (Moyes et al., 2016) is currently under study to further characterize its activity.



Figura/Figure 1.8 Amino acid sequence of the Ece1 protein. The activity of the protease Kex2p renders eight peptides named Ece1-I to VIII. The scheme highlights the signal peptide (SP) and the KR motifs (lysine-arginine) in the C-extreme of each peptide (Moyes et al., 2016).

2 Objectives





2

Objetivos

Objectives

En las últimas décadas la incidencia de la candidiasis invasora (CI) se ha incrementado drásticamente. Debido a que *Candida* es un componente habitual de la microbiota, resulta difícil diferenciar una infección de una colonización.

Nuestro grupo de investigación desarrolló una técnica de inmunofluorescencia indirecta que detecta la presencia de aquellos anticuerpos que reaccionan específicamente con la pared de los tubos germinales de *C. albicans* en pacientes con una CI. Estos anticuerpos aparecen durante un proceso invasivo y se les ha denominado CAGTA. La tasa de mortalidad de aquellos pacientes con una CI en la UCI era significativamente inferior en aquellos que presentaban títulos altos de CAGTA en suero. Basándonos en estas premisas, el primer objetivo de este trabajo fue el de caracterizar la actividad antifúngica de los

In the last decades, the incidence of invasive candidiasis (IC) has dramatically increased. Since *Candida* is a common component of human microbiota, it is difficult to differentiate invasion from colonization.

Our group of research developed an immunofluorescence indirect assay to detect the presence of those antibodies reacting specifically against the surface of the germ tubes of *Candida albicans* in patients with IC. These antibodies appear during the invasive process and they are referred to as CAGTA. The mortality rate of patients with IC at the ICU was significantly lower in those with high CAGTA-titer in serum. Based on this premise, the first goal of this study was to characterize the antifungal activity of anti-*Candida albicans* germ tube antibodies (CAGTA).



anticuerpos anti-micelio de *C. albicans* (CAGTA).

Recientemente el grupo de investigación del Dr. Naglik descubrió la Candidalisina, la primera toxina citolítica peptídica identificada en hongos patógenos humanos. Este péptido presenta un papel fundamental en el proceso infeccioso de las mucosas por parte de *C. albicans* gracias a la activación de una respuesta inmunológica a nivel epitelial. La Candidalisina deriva de una preproteína (Ece1p) formada por 271 aa, incluyendo siete motivos dibásicos lisina-arginina (KR). Estos motivos KR son reconocidos *in vitro* por la endoproteasa Kex2p del aparato de Golgi. La maduración de la Candidalisina requiere dos eventos enzimáticos: el primero implica el procesamiento de Ece1p por parte de Kex2p que posteriormente es procesada por Kex1p. En esta sección del estudio nos propusimos determinar si la actividad proteolítica requerida para la producción del péptido Candidalisina madura es dependiente de la actividad Kex1p; y por otro lado determinar la independencia de la carga-positiva del extremo C-terminal del péptido Candidalisina para promover daño a nivel epitelial.

A continuación, se detallan los objetivos de este trabajo:

1. Caracterizar la evolución de los CAGTA durante el proceso infeccioso e identificar los antígenos reconocidos

Recently, Dr Naglik's research group discovered the Candidalysin, the first cytolytic peptide toxin identified in any human fungal pathogen. This cytolytic peptide has a vital role in mucosal infection by *C. albicans* due to the activation of the epithelial immunity. This peptide is derived from a larger parental preprotein (Ece1p) which consist of 271 aa, including seven dibasic lysine-arginine (KR) motifs that are recognized *in vitro* by the Golgi complex-associated endoprotease Kex2p. Candidalysin maturation requires two individual enzymatic events. The first involves processing of Ece1p by Kex2p, yielding immature Candidalysin, which is then further processed by Kex1p. In this section of the study we decided to determine if the proteolytic processing event required for the production of mature Candidalysin is dependent upon Kex1p; and on the other hand to determine the reliance of positively-charged C-terminal K motif of wild-type *C. albicans* Candidalysin for epithelial damage induction.

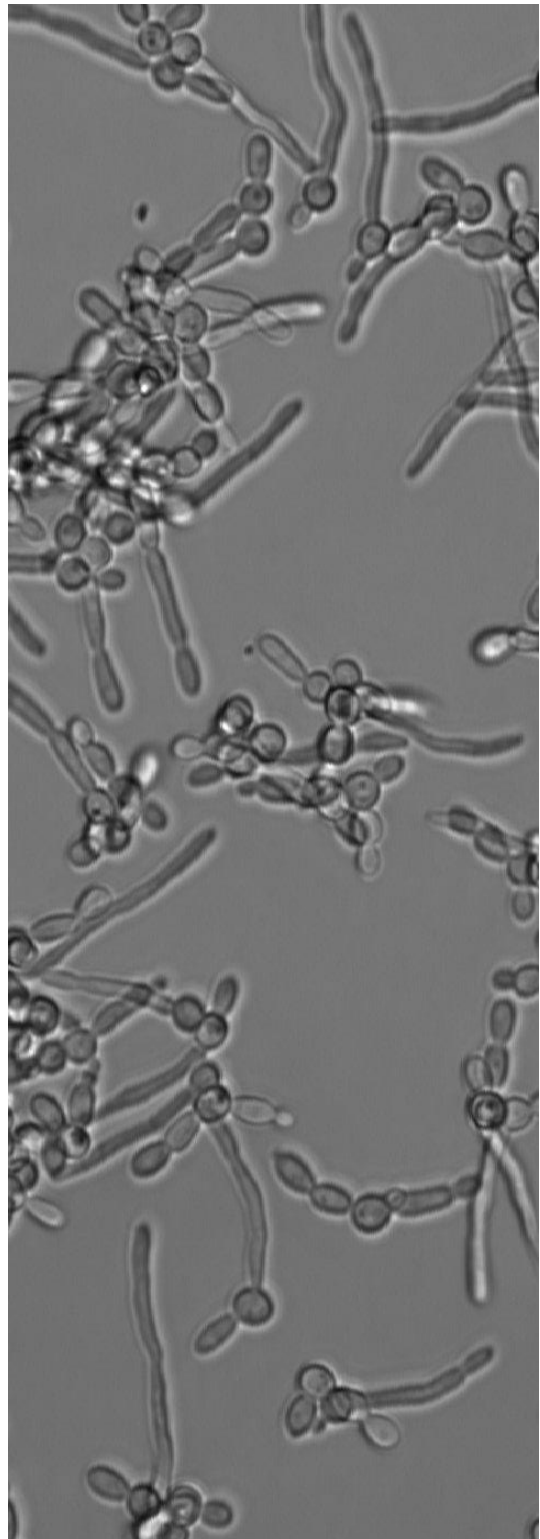
Details of the objectives of this work are listed below:

1. Characterizing the evolution of CAGTA along the candidiasis process and identifying antigens recognized by



- por los CAGTA desarrollados a partir de un modelo de CI en conejo causado por *C. albicans*.
2. Evaluar la actividad antifúngica de los CAGTA a partir de un modelo de CI en conejo determinando el efecto sobre la viabilidad, actividad metabólica y morfología de *C. albicans*.
3. Caracterizar la actividad antifúngica de los anticuerpos contra *C. albicans* desarrollados por pacientes con CI.
4. Caracterización de la actividad antifúngica de anticuerpos obtenidos a partir de un modelo de CI en conejo contra biopelículas de *C. albicans*.
5. Investigar la función de la actividad de la enzima Kex1p en la formación del péptido maduro Candidalysina.
6. Evaluación de la actividad sobre células epiteliales de diferentes variantes de Candidalysina con carga positiva, negativa o neutra debido al cambio del aminoácido en el extremo C-terminal del péptido y comparación con la Candidalysina natural.
- CAGTA developed in a rabbit model of invasive infection by *C. albicans*.
2. Evaluating the antifungal activity of CAGTA obtained in a rabbit model of IC by assessing their effect on viability, metabolic activity and morphology of *Candida albicans*.
3. Characterizing the antifungal activity of antibodies developed by patients with IC against *C. albicans*.
4. Characterizing the antifungal activity of antibodies raised in a rabbit model of IC infection against *C. albicans* biofilm.
5. Investigating the function of Kex1p activity in the generation of mature Candidalysin.
6. Evaluating the activity on epithelial cells of different variants of Candidalysin peptides with negative, positive or neutral charge amino acid substitutions at the C-terminal position of the protein and comparison to the wild-type Candidalysins.

3 Patients, Materials and Methods





3

3.1 Microorganisms and culture media

3.1.1 Microorganisms

The reference fungal and bacterial strains used in this study are listed in Tabla/Table 3.1 and Tabla/Table 3.2.

Tabla/Table 3.1 Yeast reference strains.

Identity	Source
<i>Candida albicans</i> NCPF 3153	NCPF ¹
<i>Candida albicans</i> SC5314	SC ²

¹NCPF: National Culture of Pathogenic Fungi, Bristol, U.K.

²SC: Stanford DNA Sequencing and Technology Center, Stanford, USA.

Tabla/Table 3.2 Bacteria reference strains.

Strain	Genotype	Source
<i>Escherichia coli</i> Tuner®	F ⁻ <i>ompT hdsS_B(r_B m_B) gal dcm lacY1</i> (DE3) pLacI (Cm ^R)	Novagen (USA)
<i>Escherichia coli</i> XL1-Blue	<i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44</i> <i>relA1 lac [F' proAB lacIq ZΔM15 Tn10 (Tetr)]</i> .	Agilent Technology (USA)

3.1.2 Culture media and growth conditions for *Candida*

Material

✓ **Medium 199 (m199; Sigma-Aldrich, USA)**

Media was sterilized by filtration (Φ: 0, 22 μm; Thermo Fisher Scientific, USA).

✓ **Sabouraud dextrose Broth/Agar (SDA/SDB; BD, USA)**

✓ **RPMI medium supplemented with 10% (wt/vol) FBS**

Fetal bovine serum (FBS; Gibco, USA) 10 ml

RPMI 1640 (Gibco, USA) 90 ml



Method

C. albicans was routinely grown on SDA at 30°C for 48 h. It was maintained at room temperature suspended in water, or at -80°C in SDB supplemented with 15% (vol/vol) glycerol (Panreac, Spain).

3.1.3 Culture media and growth conditions for *Escherichia coli*

Material

- ✓ Luria-Bertani (LB) broth/agar (Pronadisa, Spain)
- ✓ NZYM agar/top agar (Teknova, USA)

Method

E. coli was routinely grown on LB agar at 37°C for 24 h. For each experiment, we followed the conditions summarized in Tabla/Table 3.3

Tabla/Table 3.3 *E. coli* growing conditions

Experiment	Culture medium	Antibiotic	Temperature	Agitation r.p.m.
Protein induction	LB broth	ampicillin (50µg/ml) chloramphenicol (34µg/ml)	37°C	240
Phage technique	LB broth	tetracycline (10µg/ml)	30°C	240
	NZYM agar/top agar	--	37/42°C	--

3.2 Patients and sera

A total of 29 sera from patients hospitalized in different units of the Severo Ochoa Hospital of Madrid and Cruces University Hospital (Bizkaia), in Spain were studied. The patients were sorted into 4 groups depending on their prior diagnosis and anti-mycelia antibody titers.

- **Group I:** The group I included 8 sera from 8 patients infected by *C. albicans* with high anti-mycelia antibody titers (Tabla/Table 3.4).
- **Group II:** The group II included 7 sera from 7 patients infected by *C. albicans* with low anti-mycelia antibody titers (Tabla/Table 3.5).



- **Group III:** The group III included by 8 sera from 8 patients infected by different species of *Candida* no-*C. albicans* with high anti-mycelia antibody titers (Tabla/Table 3.6).
- **Group IV:** The group IV included 6 sera from 6 patients infected by different species of *Candida* no-*C. albicans* with low or without anti-mycelia antibody titers (Tabla/Table 3.7).

Tabla/Table 3.4 Patients sera used in this study. Group I.

Group I: <i>Candida albicans</i> and titer AM \geq 1/160							
	Unit	CAGTA (reverse titer) ^c	β -glucan ^d	α -Als3-N ^e	α -Hwp1-N ^f	α -Eno-1 ^g	Clinical isolate
1	ICU ^a	1/2560	586.25	1.4	15.05	1.76	<i>C. albicans</i>
2	ICU ^a	1/1280	>10000	3.49	12.67	11.68	<i>C. albicans</i>
3	ICU ^a	1/1280	1036.1	2.45	4.35	1.28	<i>C. albicans</i>
4	Internal medicine	1/160	10.63	1.93	1.03	1.26	<i>C. albicans</i>
5	ICU ^a	1/1280	217.02	0.86	5.37	1.53	<i>C. albicans</i>
6	Internal medicine	1/1280	1344.1	0.83	1.05	3.4	<i>C. albicans</i>
7	Geriatric	1/5120	715.23	1.2	0.41	1.67	<i>C. albicans</i>
8	REA-ICU ^b	1/320	181.76	1.62	1.14	0.81	<i>C. albicans</i>

^aICU: Intensive Care Unit.

^bREA-ICU: Units of Reanimation and Intensive Care.

^cCAGTA: reverse titer of antibodies against germ tubes of *C. albicans*. Serum dilution equal or greater than 1/160 were considered positive for IC.

^d β -glucan: values in [pg/ml] obtained from Fungitell (Fungitell® β -D-Glucan Assay, Associates of Cape Cod, Inc., USA). Values equal to or greater than 1/80 pg/ml are considered positive for fungal infection.

^e α -Als3-N: antibodies anti-N-terminus of the protein agglutinin-like sequence. Relative absorbance values obtained by the enzyme-linked immunosorbent assay (ELISA). Cut off: 1.14.

^f α -Hwp1-N: antibodies anti-N-terminus of the Hyphal wall protein 1. Relative absorbance values obtained by the enzyme-linked immunosorbent assay (ELISA). Cut off: 0.81.

^g α -Eno-1: antibodies anti-Enolase protein. Relative absorbance values obtained by the enzyme-linked immunosorbent assay (ELISA). Cut off: 2.123.

**Tabla/Table 3.5** Patients sera used in this study. Group II.

Group II: <i>Candida albicans</i> and titer AM\leq1/80							
	Unit	CAGTA (reverse titer) ^c	β -glucan ^d	α -Als3-N ^e	α -Hwp1-N ^f	α -Eno-1 ^g	Clinical isolate
1	General surgery	20	555.26	0.71	1.09	1.73	<i>C. albicans</i>
2	Internal medicine	40	271.6	0.4	0.97	2.58	<i>C. albicans</i>
3	Internal medicine	0	355.1	0.57	1.15	1.17	<i>C. albicans</i>
4	Hematology	10	885.58	0.67	1.19	1.95	<i>C. albicans</i>
5	REA-ICU ^b	40	182.63	0.94	2.37	2.09	<i>C. albicans</i>
6	Hematology	20	513.4	1.29	0.87	0.97	<i>C. albicans</i>
7	Hematology	10	10.24	0.89	1.97	1.08	<i>C. albicans</i>

*Abbreviations: see Tabla/Table 3.4

Tabla/Table 3.6 Patients sera used in this study. Group III.

Group III: <i>Candida no-Candida albicans</i> and titer AM\geq1/160							
	Unit	CAGTA (reverse titer) ^c	β -glucan ^d	α -Als3-N ^e	α -Hwp1-N ^f	α -Eno-1 ^g	Clinical isolate
1	Internal medicine	160	1888.3	1.63	1.66	1.92	<i>C. tropicalis</i>
2	ICU ^a	2560	33436	2.6	3.72	4.01	<i>C. glabrata</i>
3	General surgery	160	16611	1.76	2.22	4.85	<i>C. glabrata</i>
4	ICU ^a	160	408.73	0.55	1.17	0.9	<i>C. parapsilosis</i>
5	Hematology	160	58.13	0.74	0.85	0.97	<i>C. parapsilosis</i>
6	ICU ^a	160	53.88	1.03	1.82	2.04	<i>C. parapsilosis</i>
7	ICU ^a	320	461.94	1.36	2.94	1.88	<i>C. parapsilosis</i>
8	ICU	160	35.05	1.27	0.49	1.09	<i>C. parapsilosis</i>

*Abbreviations: see Tabla/Table 3.4

**Tabla/Table 3.7** Patients sera used in this study. Group IV.

Group IV: <i>Candida no-Candida albicans</i> and titer AM \leq 1/80							
	Unit	CAGTA (reverse titer) ^c	β -glucan ^d	α -Als3-N ^e	α -Hwp1-N ^f	α -Eno-1 ^g	Clinical isolate
1	General surgery	80	284.26	0.61	1.61	0.21	<i>C. glabrata</i>
2	ICU ^a	20	1696.4	0.92	1.12	0.94	<i>C. tropicalis</i>
3	ICU ^a	10	154.14	0.8	1.32	1.73	<i>C. parapsilosis</i>
4	Hematology	0	17.97	0.28	0.54	1.31	<i>C. parapsilosis</i>
5	Hematology	0	122.23	0.42	0.62	0.95	<i>C. glabrata</i>
6	Hematology	0	57117	0.29	0.49	0.32	<i>C. glabrata</i> / <i>C. tropicalis</i>

*Abbreviations: see Tabla/Table 3.4

3.3 Sera from with disseminated candidiasis

New Zealand White female rabbits (2-2.5kg weight) (Granja Cunicola de San Bernardo S.L. Navarra, Spain) were used to produce polyclonal sera. The rabbits were maintained at the animal facilities of the UPV-EHU (Spain) according to animal welfare ethics policy. They were injected through the ear marginal vein with 2×10^6 blastospores of *C. albicans* SC5314 suspended in 0.2 ml of sterile saline solution (day 0); inoculation was repeated on days 28, 56, 84, 112 and 154. Pre-immune sera were withdrawn prior to infection; immune sera were collected through the ear marginal vein every week after the onset of infection. Blood samples were left to clot at 4°C overnight, and sera were stored at -20°C. Serum CAGTA levels were titrated by IIF according to Moragues et al. (Moragues et al., 2004). Characteristics of sera are shown in Tabla/Table 3.8.

**Tabla/Table 3.8** Rabbit sera used in this work. Source: Dr. Pontón laboratory.

Serum	CAGTA ^a	α-BL ^b	Inoculations	Day of collection	α-14-3-3 ^c	α-Adh1 ^d	α-Als3-N ^e	α-Eno-1 ^f	α-CW-Eno ^g	α-Hwp1-N ^h	α-Met-6 ⁱ	Type infection
16706	1/1024	1/4096	2	37	2.06	1.21	2.86	6.95	5.26	18.08	1.84	Infected by <i>C. albicans</i>
16715	1/1024	1/2000	5	115	1.27	8.6	2.08	3.13	3.53	6.11	3.95	
Control	--	--	--	--	--	--	--	--	--	--	--	Pre-immune sera

^aCAGTA: reverse titer of anti-mycelium antibodies sera dilutions equal to or greater than 1/160 (sera dilution) were considered positive for IC.

^bα-BL: reverse titer of anti-blastospore antibodies.

^c α-14-3-3: antibodies anti-14-3-3 protein. Relative absorbance values obtained by the enzyme-linked immunosorbent assay (ELISA).

^dα-Adh1: antibodies anti-alcohol dehydrogenase 1 protein. Relative absorbance values obtained by the enzyme-linked immunosorbent assay (ELISA).

^eα-Als3-N: antibodies anti-N-terminus of the protein agglutinin-like sequence. Relative absorbance values obtained by the enzyme-linked immunosorbent assay (ELISA).

^fα-Eno-1: antibodies anti-Enolase 1. Relative absorbance values obtained by the enzyme-linked immunosorbent assay (ELISA).

^gα-CW-Eno: antibodies anti-cell wall enolase. Relative absorbance values obtained by the enzyme-linked immunosorbent assay (ELISA).

^hα-Hwp1-N: antibodies anti-N-terminus of the Hyphal wall protein. Relative absorbance values obtained by the enzyme-linked immunosorbent assay (ELISA).

ⁱα-Met-6: antibodies anti-methionine synthase. Relative absorbance values obtained by the enzyme-linked immunosorbent assay (ELISA).

3.4 Production of *Candida albicans* yeast cells heat-inactivated

Material

✓ Phosphate buffered saline (PBS) (10X)

Na ₂ HPO ₄ (Panreac, Spain)	10.2 g
NaH ₂ PO ₄ (Panreac, Spain)	3.86 g
NaCl (Panreac, Spain)	85 g
Distilled water	up to 1 l

pH adjusted to 7.2

It was diluted tenfold with water and sterilized in the autoclave at 121°C for 15 min whether necessary

✓ Sabouraud dextrose agar (SDA)



Method

C. albicans strains were grown on SDA at 24°C for 48 h. Yeast cells were harvested with PBS and washed three times by centrifugation at 2500 rpm for 5 min. The pellet was suspended in sterile PBS (around 10¹¹ cell/ml) and the blastospores were killed by heating at 60°C for 2 h in a water bath. Dead cells were washed 3 times with PBS, counted in a hemacytometer and suspended at 10¹⁰ cell/ml. Cell inactivation was evaluated through the spread plate method on SDA and incubation at 24°C for 48 h.

3.5 Production of *Candida albicans* mycelia

Material

- ✓ **Medium 199 (m199)**
- ✓ **PBS**
- ✓ **Sabouraud dextrose agar (SDA)**

Method

Two or three colonies of *C. albicans* yeast cells grown on SDA at 24°C for 48h were inoculated in 10 ml of m199 overnight at 24°C with shaking (120 rpm). Yeast cells were harvested by centrifugation at 2500 rpm for 5 min and inoculated into 4 volumes of m199 pre-warmed to 37°C and incubated for 3.5 h at 120 rpm. Germ tubes were harvested by filtration through a sintered glass filter, washed with PBS, and stored at -20°C until used.

3.6 Serum fractionation, quantification and purification of immunoglobulins (IgG)

Material

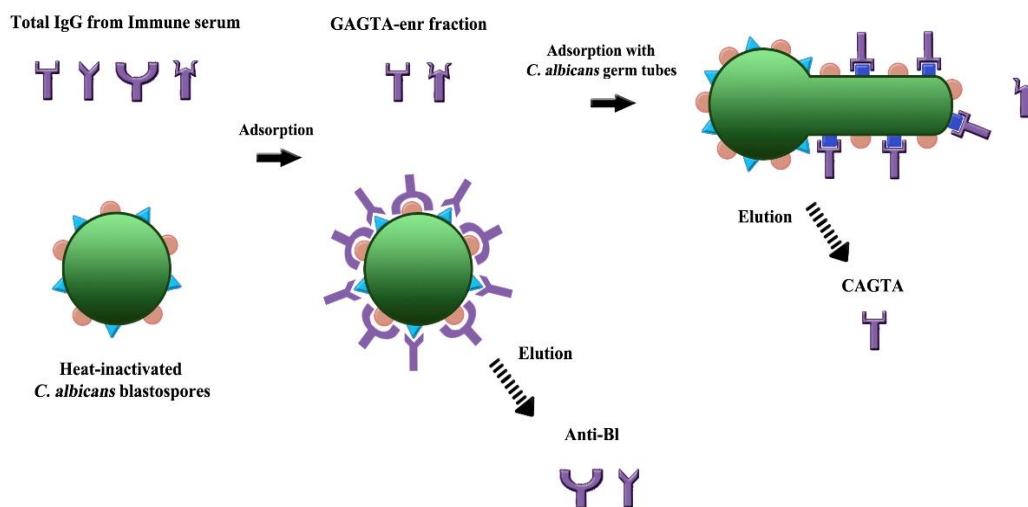
- ✓ **2.5 M sodium iodide**

Sodium iodide (Sigma-Aldrich, USA)	1.87 g
PBS	1 l



Method

Immune sera were obtained from White New Zealand rabbits infected by *C. albicans* SC5314 as previously described (section 3.3) and from patients with IC (section 3.2), and were divided into the following fractions: total IgG fraction from immune serum (total-IgG), CAGTA enriched fraction (CAGTA-enr), anti-blastospore fraction (anti-BL) and purified CAGTA, in order to run different assays (Figura/Figure 3.1).



Figura/Figure 3.1 Scheme of anti-mycelia antibodies (CAGTA) from human or rabbit sera infected by *Candida* spp.

Total-IgG from immune serum included antibodies against the superficial antigens of *C. albicans* blastospores (anti-BL) and germ tubes (CAGTA), together with non-specific antibodies. CAGTA enriched IgG serum fraction (CAGTA-enr) was obtained after absorbing whole serum with an equal volume of heat-killed *C. albicans* blastospores (10^{10} cell/ml of PBS) for 2h at room temperature, followed by centrifugation at 2500 rpm for 5 min to retire anti-BL antibodies adsorbed on the cells (Moragues et al., 2004). The IgG class antibodies were purified with Melon™ Gel IgG Spin Purification Kit (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. Purified CAGTA were obtained after incubating the CAGTA-enr serum fraction with an equal volume of a pellet of *C. albicans* germ tubes at room temperature for one hour with gentle agitation. Then the cell suspension was centrifuged and the pellet was washed with PBS (Sigma-Aldrich, USA). The adsorbed antibodies were eluted from the mycelia surface in 5 ml of 2.5 M sodium



iodide (Sigma-Aldrich, USA) in PBS by gentle shaking at room temperature for one hour and then mycelia were eliminated by centrifugation. The supernatants containing the eluted CAGTA were dialyzed (MWCO 12.000–14.000 Da; Medicell International, London, UK) against PBS and concentrated with polyethylene glycol 20.000 (Merck Millipore, Germany) (Sáez-Rosón et al., 2014). The protein concentration of serum fractions was estimated with Pierce™ Coomassie Plus (Bradford) Assay Kit (Thermo Fisher Scientific, USA) according to the manufacturer's instructions.

3.7 Serum titration for CAGTA

Material

✓ **0.5 M Carbonate bicarbonate buffer at pH 9**

Na ₂ CO ₃ (Sigma-Aldrich, USA)	1.59 g
NaHCO ₃ (Sigma-Aldrich, USA)	2.93 g
NaN ₃ (Sigma-Aldrich, USA)	0.2 g
Distilled water	up to 1l
pH adjusted to 9	

✓ **4% (vol/vol) Formaldehyde in PBS**

Formaldehyde 36-38% (Sigma-Aldrich, USA)	10 ml
PBS	90 ml

✓ **Anti-human IgG (whole molecule)-FICT antibody (Sigma-Aldrich, USA)**

✓ **Anti-rabbit IgG (whole molecule)-FICT antibody (Sigma-Aldrich, USA)**

✓ **Buffered-glycerol**

Glycerol (Panreac, Spain)	90 ml
0.5 M Carbonate bicarbonate buffer pH 9.0	10 ml

✓ **Heat-inactivated blastospores**

✓ **PBS-Evans blue-Tween 20 buffer (PBS-TE)**

Evans Blue (Merck Millipore, Germany)	0.5 g
Tween 20 (Sigma-Aldrich, USA)	0.5 ml



PBS

up to 1 l

Method

Sera titration was carried out through the indirect immunofluorescent technique previously described by Moragues et al. (Moragues et al., 2004). Briefly, in order to eliminate anti-mannan antibodies, the serum was diluted $\frac{1}{4}$ in PBS and then it was adsorbed with 4 volumes of a suspension of heat-inactivated *C. albicans* blastospores (10^{10} cell/ml) for 1 h at room temperature. Adsorbed sera were centrifuged at 2500 rpm for 5 min. Serial dilutions in PBS of the supernatants were tested against germ tubes of *C. albicans* immobilized on multiwell glass slides that had been prepared in advance according to the following instructions.

Ten microliters of germ tube suspension (approximately 10^4 germ tubes in PBS-formaldehyde) were added to each well (Thermo Fisher Scientific, USA) and left to dry in the air. Mycelia were fixed by submerging the slide into acetone, and then they were washed 3 times with PBS for 1 min with gentle shaking. Once dried, the slides were stored at 4°C until used.

Twelve microliters of the serially diluted adsorbed sera were added to each well. Regarding rabbit IgG, a concentration 12.5-200 μ g/ml was added in each well. A positive control was included in each assay. Slides were incubated in a wet chamber at 37°C for 30 min. Slides were washed with PBS for 5 min with gentle shaking and then left to dry. Eleven microliters of the secondary antibody (FITC conjugated anti-human or anti-rabbit IgG; Sigma-Aldrich, USA) diluted 1/150 in PBS-TE was added to each well. Slides were incubated at 37°C for 30 min, washed with PSB, and left to dry in the air. The slides were mounted with buffered-glycerol and were examined with an AZ100 microscope (Nikon, Tokyo, Japan), provided by Analytical and High-Resolution Microscopy in Biomedicine Service, SGIker of the UPV-EHU, or Eclipse80i equipped for both phase-contrast optics and epifluorescence (with a FITC filter).



3.8 Protein assays

3.8.1 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Material

✓ **10% (wt/vol) APS**

APS (ammonium persulfate; BioRad, USA)	10 g
Distilled water	100 ml

APS is prepared shortly prior to use

✓ **10 % (wt/vol) SDS**

SDS (sodium dodecyl sulfate; BioRad, USA)	10 g
Distilled water	up to 100 ml

✓ **Running Buffer**

Trizma base (Sigma-Aldrich, USA)	36.3 g
Distilled water	up to 200 ml

pH adjusted to 8.8

✓ **Running gel SDS -PAGE (10%)**

Running buffer	2.5 ml
Acrylamide/bis-acrylamide 40% (29:1 ratio) (Pronadisa, Spain)	2.475 ml
Water distilled	4.875 ml
SDS	0.1 ml
APS	50 µl
TEMED (BioRad, USA)	5 µl

TEMED and APS solutions have to be added after degassing the solution.

✓ **Sample loading buffer (5X)**

1M Tris-HCl (pH 6.8)	2.5 ml
Glycerol (Panreac, Spain)	2 ml
SDS	4 ml
Bromophenol blue (Sigma-Aldrich, USA)	1 mg

Shortly before use, add 5% (vol/vol) β-mercaptoethanol (BioRad, USA)



✓ **Stacking Buffer**

Trizma base (Sigma-Aldrich, USA)	3 g
Distilled water	up to 50 ml
pH adjusted to 6.8	

✓ **Stacking gel SDS-PAGE (3%)**

Stacking buffer	1.25 ml
Acrylamide/bis-acrylamide 40% (29:1 ratio)(Pronadisa, Spain)	488 µl
Distilled water	3.21 ml
SDS	75 µl
APS	25 µl
TEMED (BioRad, USA)	5 µl

TEMED and APS solutions have to be added after degassing the solution.

✓ **Tris-glycine SDS buffer (5X)**

Trizma Base (Sigma-Aldrich, USA)	9 g
Glycerol (Panreac, Spain)	43.2 g
SDS (BioRad,USA)	3 g
Distilled water	up to 600 ml

Method

The protein separation using polyacrylamide gels in denaturing conditions was carried out according to the standard procedure of Laemmli and colleagues (Laemmli, 1970). The samples were diluted in sample loading buffer and heated at 100°C for 5 min.

The samples were supplemented with ¼ volume of sample loading buffer and β-mercaptoethanol was added up to 5%. The samples were heated in a boiling water bath for 5 min. Twelve microliters of each sample were loaded into their corresponding wells. Five microliters of a protein Ladder (BenchMark™, Invitrogen, USA) was used as molecular weight markers reference. Electrophoresis was run at 200 V for 45 min using a MiniProtean III system (BioRad, USA).

The proteins separated by SDS-PAGE, were stained using Coomassie blue dye (section 3.8.2).



3.8.2 Coomassie blue staining

Material

✓ **Coomassie solution**

Coomassie blue (BioRad, USA)	1.2 g
Methanol (Panreac, Spain)	500 ml
Acetic acid (Sigma-Aldrich, USA)	100 ml
Distilled water	up to 1 l

✓ **Destaining solution I**

Methanol (Panreac, Spain)	500 ml
Acetic acid (Sigma-Aldrich, USA)	100 ml
Distilled water	up to 1 l

✓ **Destaining solution II**

Methanol (Panreac, Spain)	50 ml
Acetic acid (Sigma-Aldrich, USA)	70 ml
Distilled water	up to 1 l

Method

Coomassie staining is able to detect protein bands containing $\geq 0.2 \mu\text{g}$ of protein after SDS-PAGE. The gels were incubated with Coomassie blue solution for 1 h with gentle agitation. The excess of colorant was removed and, the gels were treated with destaining solution I for 1 hour with gentle shaking. Finally, after discarding the solution I, destaining solution II was added in order to rehydrate and store the gels.

3.8.3 Membrane Electrotransfer and Ponceau red staining

Material

✓ **Acetic acid (Sigma-Aldrich, USA)**

✓ **Methanol (Panreac, Spain)**

✓ **Ponceau solution**

Ponceau red (Sigma-Aldrich, USA)	1 g
Acetic acid (Panreac, Spain)	50 ml



Distilled water	up to 1 l
✓ Transfer buffer	
Trizma Base (Sigma-Aldrich, USA)	3.02 g
Glycerol (Panreac, Spain)	11.26 g
Methanol	100 ml
Distilled water	up to 1 l

Method

After SDS-PAGE, the proteins were transferred onto PVDF membranes (PVDF-Immobilon, Merck Millipore, Germany) followed by a Western Blot (WB) using specific antibodies. The membranes were treated with methanol for 5 min and then submerged into transfer buffer for 5 min. Chromatography papers were also soaked in transfer buffer immediately prior to use.

The gel and the membrane were sandwiched between 3 pieces of chromatography paper on either side. The sandwich was placed on the platinum cathode semi-dry Fast Blot System (Biometra, Germany) and then the anode plate was carefully placed on top. The transfer was run for 1 h with a constant current of 5 mA per cm² of gel. The protein transfer was verified using the Ponceau red staining. The membranes were dyed with the Ponceau solution for 2 min and then membranes were washed with 5% (vol/vol) acetic acid solution.

3.8.4 Western blot analysis

Material

✓ 8% (wt/vol) milk in TBS	
Skim milk powder (Merck Millipore, Germany)	8 g
TBS	100 ml
✓ Primary antibody	
Anti-HSV•Tag® (GenScript, USA) 1:200 in TBS-milk	
✓ Secondary antibody	
Anti-rabbit IgG-POX (Sigma-Aldrich, USA) 1:300 in TBS-milk	
✓ TBS (tris-buffered saline)	
Trizma Base (Sigma-Aldrich, USA)	12 g



NaCl (Panreac, Spain)	9 g
Distilled water	up to 1 l
pH adjusted to 7.3	

Method

PVDF membranes were incubated with the primary antibody in TBS in a humid chamber at 37°C for 1 h with gentle shaking. After washing three times with TBS, the membranes were incubated with the secondary antibody in TBS-milk under the same conditions. After washing with TBS, the membranes were soaked with the Clarity™ Western ECL Substrate kit (BioRad, USA). The reactions were visualized with the Chemidoc Quantity One system (BioRad, USA).

3.8.5 Determination of protein concentration

Total protein concentration was determined using the Coomassie Plus Protein Assay Reagent Kit® (Thermo Fisher Scientific, USA), according to the manufacturer's instructions. The technique was described by Bradford and colleagues (Bradford, 1976). The absorbance was read at 595 nm using a Microplate Autoreader Bio-Tek Instruments (Bio-Tek®, USA).

3.9 Recombinant antigens

A recombinant-protein based enzyme linked immunosorbent assay (ELISA) using different antigens from the cell wall of the mycelium of *C. albicans* was developed. The recombinant proteins were 14-3-3, Adh1, Als3-N, Eno-1, Hwp1-N, Met-6 and the antigenic component CW-Eno, whose future application for the diagnosis of invasive candidiasis has been discussed in our laboratory.

E. coli strains containing the recombinant plasmid codifying for 14-3-3, Eno-1, Hwp1-N, Met-6 and Als3-N were developed in our laboratory. Dr. Fernando Hernando of the Department of Immunology, Microbiology and Parasitology of the UPV-EHU kindly supplied the Adh1 producing strain. CW-Eno was purified from DTT cell wall extract of germ tubes (DTT-GT) of *C. albicans*.



3.9.1 Recombinant protein induction

Material

✓ **1 M IPTG**

IPTG (isopropyl β -D-1-thiogalactopyranoside; Sigma-Aldrich, USA) 2.383 g

Distilled water 10 ml

✓ **LB broth/agar**

✓ **PBS**

Method

Escherichia coli (DE3) pLacI (Novagen, Darmstadt, Germany) was used as the host strain for the expression of the recombinant plasmid (pTriEx-1[®] vector, Novagen, USA). *E. coli* cells were grown in LB broth, supplemented with carbenicillin (50 μ g/ml) and chloramphenicol (34 μ g/ml) in order to make the medium selective for the recombinant strains, for 18 h at 37°C and 250 rpm.

The following day, 4 ml of these cultures were inoculated in 500 ml of fresh LB-medium supplemented with the same antibiotics to achieve an OD₆₀₀ 0.1-0.2. Then they were incubated until they reached an OD₆₀₀ 0.7-0.8. At this moment, the expression of the recombinant Hwp1-N protein was induced with 0.5 mM IPTG. After 3.5 h of induction, cells were harvested by centrifugation at 4500 rpm for 10 min and suspended in sterile PBS. The extract was stored at -20° C for 24 h and was treated with a commercial kit Bugbuster[®]HisBind[®]Purification (Novagen, USA) according to the manufacturer's instructions. In order to check the expression of the recombinant proteins, 1 ml of culture was centrifuged at 13200 rpm during 1 min. The pellet was suspended in 100 μ l of PBS and the extract was thawed/frozen three times in order to obtain physically lysed cells. Protein extracts were checked for the presence of the recombinant Hwp1-N fragments by SDS-PAGE. The electrophoresis was followed by transference to a PVDF membrane and western blotting using the monoclonal antibody HSV-Tag[®] (GenScript, USA).



3.9.2 Protein purification

Method

The recombinant protein was purified using the commercial Bugbuster®HisBind®Purification kit (Novagen, USA). The protein carries a six histidine (*His*) residue tail that binds to Ni²⁺ cations immobilized on resin.

Previously to the purification, the column was filled with 5 ml of resin and it was purified following manufacturer's instructions.

The eluted fractions containing the purified polypeptides were pooled and dialyzed against PBS (1 ml sample vs 100 ml PBS, buffer change every 2 h, 3 times). The protein in the dialysis bag was concentrated using polyethylene glycol 20.000 (Merck Millipore, USA). The protein concentration of the sample was estimated using the Coomassie Plus Proteins assay (section 3.8.5) and it was stored at -20°C until use.

3.10 Detection of antibody in sera by ELISA

Material

✓ **0.5 M Carbonate-bicarbonate buffer pH 9.6**

✓ **0.5 M Stop solution (H₂SO₄)**

H₂SO₄ (sulfuric acid; Prolabo, Spain) 2.6 ml

Distillated water up to 100

✓ **0.05%(vol/vol) Tween20 in PBS**

PBS 100 ml

Tween 20 (Sigma-Aldrich, USA) 0.05 ml

✓ **1%(wt/vol) BSA in PBS**

BSA (bovine serum albumin; Sigma-Aldrich, USA) 1g

PBS 100 ml

✓ **Anti-rabbit-IgG POX (Sigma-Aldrich, USA)**

✓ **PBS**✓ **PBS-BT**

0.05% (vol/vol) Tween 20 (Sigma-Aldrich, USA)	0.05 ml
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1% (wt/vol) BSA (Sigma-Aldrich, USA)	1 g
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PBS	100 ml
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✓ **Substrate solution for ELISA detection**

0.05 M Citric acid (BioRad, USA)	4.86 ml
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0.1 M Na ₂ HPO ₄ (Panreac, Spain)	5.14 ml
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OPD (o-Phenylenediamine; Sigma-Aldrich, USA)	5 mg
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30 % (vol/vol) H ₂ O ₂	5 µl
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Add H₂O₂ just prior to use

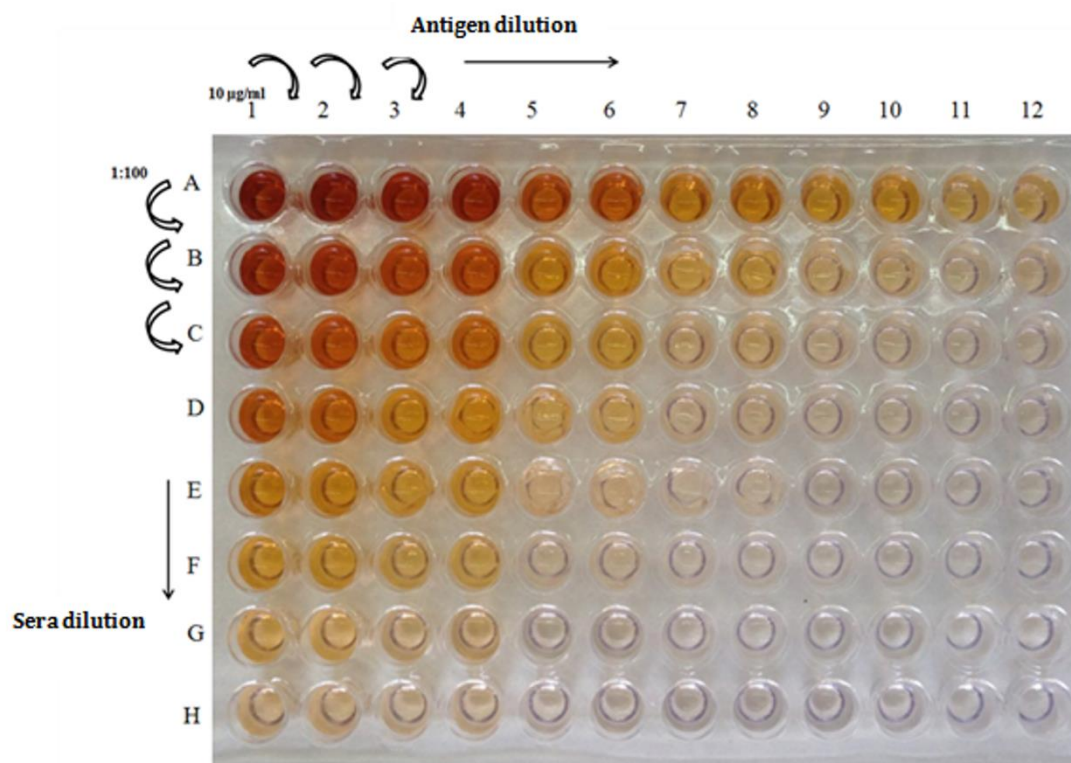
Method

Microtiter vinyl plates (Costar, USA) were coated with the purified recombinant proteins 14-3-3, Adh1, Als3-N, Eno-1, Hwp1-N, Met-6, or with the CW-Eno. One hundred microliters of protein diluted in carbonate buffer at the concentration shown in Tabla/Table 3.9 and Tabla/Table 3.10 were added to each well and the plates were incubated at 4°C overnight. Then, the wells were emptied, washed with 200 µl of PBS, and blocked with 200 µl of PBS-BSA at 37°C for 1 h. Sera diluted in PBSBT at the concentration established in the Tabla/Table 3.9 and Tabla/Table 3.10 were assayed in triplicate (100 µl per well). After 1 h of incubation at 37°C, the wells were emptied and washed three times with 200 µl of PBS-Tween 20. One hundred microliters of peroxidase conjugated anti-rabbit or anti-human anti IgG (Sigma-Aldrich, USA) diluted 1:1000 in PBSBT were added to each well and the plates were incubated at 37°C for 1 h. After washing, each well was filled with 100 µl of the substrate solution. The plates were incubated at room temperature for 30 min in the dark. The reaction was stopped by adding 50 µl per well of 0.5 M sulfuric acid and the absorbance was measured at 495 nm using an ELISA Microplate Autoreader Bio-Tek Instruments (Bio-Tek®, USA).



3.10.1 Checkerboard

To optimize the conditions of ELISA, a checkerboard titration was carried for every protein. One hundred microliters of double-dilution antigens solution, starting at 10 µg/ml of bicarbonate buffer, were added from the left column to the right one (1 to 12) in order to fix the antigen. In the second step, serial double-dilutions of serum, starting at 1:100 were distributed from the upper row down to G row. H row was left without serum antibody and served as a background control (Figura/Figure 3.2). Reacting antibodies were incubated with the secondary antibody (anti-human or anti-rabbit POX conjugate) and then revealed with the substrate solution. The reaction was stopped with 50 µl of 0.5 M sulfuric acid and the $A_{490\text{ nm}}$ was registered. The combination of antigen concentration and serum dilution that gave the best signal-to-noise ratio was selected for every antigen.



Figura/Figure 3.2 ELISA checkerboard.

3.10.2 Standardization of ELISA

A reference serum was included in each ELISA assay in order to normalize the results and verify the reproducibility of our assays. For that purpose, a positive and



a negative control sera were selected based on their CAGTA positive and negative results. Absorbance values were divided by that of the negative reference sera. All the assays were run in triplicate. The cutoff value for every protein was defined as the mean relative absorbance values of sera of patients without evidence of candidiasis plus two times the standard deviation of the mean. Relative absorbance values above the cutoff were considered positive whereas results under the cutoff were negative.

Tabla/Table 3.9 ELISA conditions for rabbit sera

Recombinant protein	Protein concentration [µg/ml]	Serum dilution	Anti-rabbit IgG POX concentration	Cut-off value
14-3-3	0.625	1/100	1/1000	2.33
Adh-1	1.25	1/200	1/1000	1.61
Als3-N	1.25	1/200	1/1000	1.79
Eno-1	5	1/400	1/1000	2.97
Eno-pared	5	1/400	1/1000	2.02
Hwp1-N	0.078	1/800	1/1000	2.33
Met-6	5	1/800	1/1000	2.53

Tabla/Table 3.10 ELISA conditions for human sera.

Recombinant protein	Protein concentration [µg/ml]	Serum dilution	Anti-human IgG POX concentration	Cut-off value
14-3-3	0.31	1/200	1/1000	2.21
Adh-1	2.5	1/200	1/1000	2.1
Als3-N	0.13	1/200	1/1000	1.14
Eno-1	1.25	1/400	1/1000	1.23
Eno-pared	0.05	1/400	1/1000	1.54
Hwp1-N	0.13	1/400	1/1000	0.81
Met-6	1.25	1/100	1/1000	2.46

3.11 Evaluation of CAGTA activity against *Candida albicans* planktonic cells

The effect of antibodies on *C. albicans* was evaluated by measuring the cell metabolic activity using the colorimetric XTT and colony forming units (CFU) assays.



3.11.1 Determination of *Candida albicans* metabolic activity: XTT assay

Material

- ✓ **Human IgG (Sigma-Aldrich, USA)**
- ✓ **PBS**
- ✓ **PMS solution**

PMS (1-methoxy phenazine methosulfate; Sigma-Aldrich, USA) 0.32 mg

Ultrapure water 1 ml

Solution was sterilized by filtration (Φ : 0.45 μ m; Minisart, Sartorius, Spain)

- ✓ **Rabbit IgG (Sigma-Aldrich, USA)**
- ✓ **Sabouraud broth**
- ✓ **XTT solution**

XTT (2.3-Bis (2-methoxy-4-nitro-5-sulfohenyl)-2H-tetrazolium-5-carboxanilide; Sigma-Aldrich, USA) 0.75 mg

PBS 1 ml

Solution was sterilized by filtration (Φ : 0.45 μ m; Minisart, Sartorius, Spain)

Method

Candida albicans blastospores were grown in Sabouraud broth overnight at 30°C in an orbital shaker with 120 rpm. Yeast cells were harvested with PBS and washed three times by centrifugation at 2500 rpm for 5 min. The pellet was suspended in fresh Sabouraud broth and a suspension of *C. albicans* (10^6 cell/ml) (Ramage, Vande Walle, Wickes, & Lopez-Ribot, 2001) was distributed (50 μ l per well) in a U-bottom 96-well plate (Costar, USA). Each well was supplemented with 50 μ l of Sabouraud broth containing Abs to reach different final concentrations (50, 100 or 200 μ g/ml). Plates were incubated at 37°C for 2.5 h with gentle shaking. Afterwards plates were centrifuged at 2500 rpm, the supernatant was discarded and the metabolic activity of cells was estimated with the XTT assay, according to the protocol of Henriques et al. (Henriques, Azeredo, & Oliveira, 2006). Briefly, each well was supplemented with 90 μ l of XTT solution and 10 μ l of PMS solution, and plates were incubated at 37°C for 2 h. Then plates were shaken and the absorbance was read at 492 nm using the



Microplate Autoreader Bio-Tek Instruments (Bio-Tek®, USA). Two different controls were included in every experiment, one without antibodies, and a second one with an equivalent concentration of an irrelevant non-specific rabbit or human IgG.

3.11.2 Determination of *Candida albicans* viability: colony forming units (CFU) assay

Material

- ✓ PBS
- ✓ Sabouraud dextrose agar (SDA)

Method

Similarly to the XTT assay, after incubating yeast cells with Abs at 37°C for 2.5 h with gentle shaking, each well content was homogenized and 50 µl were inoculated and incubated onto SDA at 37°C for 48 h. Two different controls were included in every experiment, one without antibodies, and a second one with an equivalent concentration of an irrelevant non-specific rabbit or human IgG.

3.12 Evaluation of CAGTA activity against *Candida albicans* biofilm

Material

- ✓ 33% (vol/vol) acetic acid

Acetic acid (Sigma-Aldrich, USA) 33 ml

Distilled water 67 ml

- ✓ 1% (vol/vol) CV solution

CV (crystal violet; Merck Millipore, USA) 1 ml

Distilled water 99 ml

- ✓ CAGTA from rabbit 20-160 µg/ml

- ✓ Menadione solution



Menadione (Sigma-Aldrich, USA)	0.1 mM
Acetone $\geq 99.8\%$ (VWR, Spain)	1 ml

Solution was sterilized by filtration (Φ : 0.45 μm ; Minisart. Sartorius, Spain)

- ✓ **Methanol (Panreac, Spain)**
- ✓ **PBS**
- ✓ **RPMI supplemented with 10% (vol/vol) FBS**

RMPI 1640 (Gibco, USA)	900 ml
Heat-inactivated FBS (fetal bovine serum; Sigma-Aldrich, USA)	100 ml

- ✓ **Sabouraud dextrose agar (SDA)**
- ✓ **XTT solution**

XTT (2.3-Bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide; Sigma-Aldrich, USA)	0.5 mg
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PBS	1 ml
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Solution was sterilized by filtration (Φ : 0.45 μm ; Minisart. Sartorius, Spain)

Method

The day before each experiment, *C. albicans* was inoculated onto SDA plates and incubated at 37°C. Blastospores were harvested, washed, suspended at 10⁶ cell/ml in RPMI-FBS and distributed (100 μl /well) into flat-bottom 96-well plates (Corning, USA). Plates were incubated at 37°C and, according to the protocols shown below, CAGTA or irrelevant Abs were added at different times in order to test their effect at different stages of the biofilm formation process.

Protocol I: effect on biofilm formation. Irrelevant IgG or CAGTA (20-160 $\mu\text{g}/\text{ml}$) were added at time zero, and the effect on biofilm was checked after 24 h of incubation.

Protocol II: effect on biofilm maturation. Irrelevant IgG or CAGTA (20-160 $\mu\text{g}/\text{ml}$) were added to a 24 h-old biofilm and their effect was assessed after an additional 24 h incubation period at 37°C.



Protocol III: effect on *C. albicans* adhesion. *C. albicans* cells were exposed to 80 µg/ml of CAGTA for the first 90 min, then washed and incubated without antibodies up to 24 h, to allow biofilm formation.

Protocol IV: effect on biofilm development. *C. albicans* cells were incubated for 90 min at 37°C, and then supplemented with CAGTA 80 µg/ml and incubated up to 24 h prior to biofilm assessment.

For XTT assay, the media was discarded and wells were supplemented with 90 µl of XTT solution and 10 µl of menadione solution. Then, plates were incubated at 37°C for 2 h and A₄₉₂ nm was registered (Mazaheritehrani et al., 2014; Pierce, Uppuluri, Tummala, & Lopez-Ribot, 2010). For CV assay, the media was discarded and wells were washed 3 times with 200 µl of PBS and then air dried for 5 min. After fixation by 100 µl of methanol for 20 min, samples were stained with 100 µl of CV solution for 5 min. Afterwards, each well was washed 3 times with 200 µl of distilled water and then 33% (vol/vol) acetic acid (100 µl/well) solution was added. After 10 min, A₅₄₀ nm was registered (Mazaheritehrani et al., 2014).

Two negative controls were included as explained for the planktonic cells experiments.

3.13 Microscopy analysis

Material

✓ **2% (vol/vol) glutaraldehyde (fixing solution)**

Glutaraldehyde solution 25% (Merck Millipore. USA)	1 ml
Sorenson's phosphate buffer	5 ml
Distilled water	up to 10 ml

✓ **6% (wt/vol) sucrose**

Sucrose (Sigma-Aldrich, USA)	6 g
Sorenson's phosphate buffer	50 ml
Distilled water	50 ml

✓ **DiBAC stock solution**



DiBAC (bis-(1,3-dibutylbarbituric acid)trimethine oxonol; Molecular Probes, USA)	25 mg
Ethanol 100% (Prolabo, Spain)	1 ml
✓ CFDA stock solution	
CFDA (5(6)-carboxyfluorescein diacetate, succinimidyl ester; Molecular Probes, USA)	5 mg
DMSO (dimethyl sulfoxide; Sigma-Aldrich, USA)	1 ml
✓ Ethanol (Merck Millipore, USA)	
✓ Hexamethyldisilazane (Electron Microscopic Science, USA)	
✓ MOPS buffer pH 7	
MOPS (Sigma-Aldrich, USA)	2.0926 g
Distilled water	100 ml
pH adjusted to 7	
✓ MOPS buffer pH 3	
Citric acid (BioRad, Spain)	0.96 g
MOPS (Sigma-Aldrich, USA)	2.0926 g
Distilled water	100 ml
pH adjusted to 3	
✓ Sabouraud dextrose broth/agar (SDB/SDA)	
✓ Sorenson's phosphate buffer	
Na ₂ HPO ₄	11.5 g
NaH ₂ PO ₄	2.96 g
Distilled water	up to 0.5 l
pH adjusted to 7.2-7.4	
✓ RPMI-FBS	



3.13.1 Electron microscopy analysis

Candida albicans germ tubes and blastospores were grown in SDB with CAGTA 40 µg/ml for 2.5 h at 37°C, and biofilms were formed in the RPMI-FBS with CAGTA 80 µg/ml for 24 h at 37°C. Then, cells were washed in Sorenson's phosphate buffer, treated with fixing solution at RT for 1 h, and washed three times with sucrose. Cells were dehydrated using graded concentrations of alcohol (50, 70 and 100% vol/vol) for 5 min each. Air-drying of samples was carried out by rapid evaporation with hexamethyldisilazane (Electron Microscopy Sciences, USA); two washes for 5 min. Negative controls were run without CAGTA. Samples for electron microscopy were finally processed at the Analytic and High Resolution Microscopy in Biomedicine Service (SGIker, UPV-EHU).

3.13.2 Fluorescent analysis

Method

Cell viability of *C. albicans* after incubation with 20 µg/ml of purified CAGTA was assessed with the fluorescent dyes DiBAC, which stains dead cells, and CFDA, which stains live cells, following the method described by Bowman et al (Bowman et al., 2002). Grow conditions were the same indicated for XTT assay of planktonic *C. albicans* cells.

Photographs were taken using AZ100 microscope (Nikon, Tokyo, Japan), provided by the Analytical and High-Resolution Microscopy in Biomedicine Service (SGIker, UPV-EHU) equipped for both phase-contrast optics and epifluorescence. Images were assembled through Image Fiji software (Version 2.0).

3.13.3 Optical microscopy analysis

Method

Cell growth and morphology of *C. albicans* treated with total-IgG or CAGTA-enr fraction (6.25-200 µg/ml) using flat bottom 96-well microtitration plates (Iwaki, Japan) were assessed by optical microscopy. Grow conditions were the same indicated for XTT assay of planktonic *C. albicans* cells. A control group without



treatment was run for every experiment. A second control was established with cells exposed to equivalent concentrations of a commercial irrelevant rabbit IgG (Sigma-Aldrich, USA).

Photographs were taken using a confocal microscope Olympus Fluoview FV500 (Tokyo, Japan) in Analytic and High Resolution Microscopy in Biomedicine Service (SGIker, UPV-EHU). Images were assembled through Image Fiji software (Version 2.0).

3.14 Statistical analysis

The statistical analysis was performed using the *GraphPad Prism* software (version 6, *GraphPad Software, Inc., USA*) and *Sigma Plot* software (version 12.5, *Systat Software Inc., USA*). Variables were analyzed with *one-way ANOVA* or *Student's t test*. *P* values < 0.05 were considered statistically significant. All assays were performed three times and each assay was assessed in triplicate.

3.15 Screening of a *Candida albicans* cDNA library

The screening of a cDNA library from *C. albicans* SC5314 (Birse et al., 1993) was conducted using CAGTA. Dr. W.A. Fonzi and Dra P. Sundstrom (Georgetown University, Washington, USA) kindly gave an aliquot of the library. The cDNA library was prepared from RNA of *C. albicans* SC5314 exhibiting hyphal morphology using the λ ZapII vector.

3.15.1 cDNA library amplification

Material

✓ **0.2% (wt/vol) maltose (Sigma-Aldrich, USA)**

✓ **10 mM MgSO₄**

MgSO₄ (Panreac, Spain)

4.92 g

Distilled water

up to 20 ml

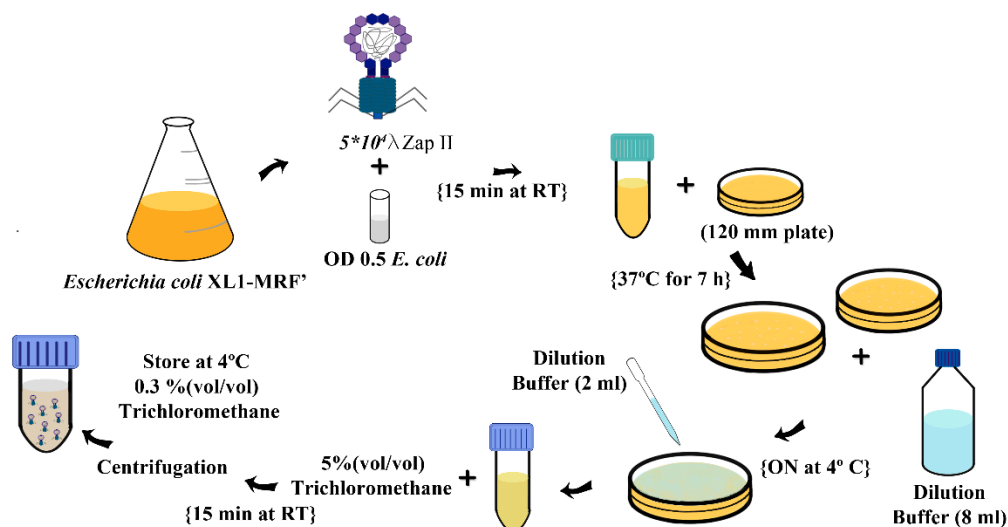
✓ **Chloroform (Merck Millipore, USA)**



- ✓ **Dilution buffer (SM Buffer; Teknova, USA)**
- ✓ **Luria-Bertani (LB) broth/agar**
- ✓ **NZYM Agar/top Agar (Teknova, USA)**

Method

Amplification of libraries prepared in lambda vectors was necessary to make a large and a stable quantity of a high-titer stock of recombinant phages. A single colony of *Escherichia coli* XL1-Blue MRF' grown overnight in LB agar supplemented with tetracycline (10µg/ml; filtered Φ : 0.22 µm; Sigma-Aldrich, USA), was inoculated into 50 ml of LB broth supplemented with 10 mM MgSO₄ and 0.2% (wt/vol) maltose (filtered Φ : 0.22 µm). The suspension was incubated overnight at 30°C and 240 rpm. Afterwards, the cells were spun down at 3500 rpm for 10 min and then cells were suspended and adjusted to an OD₆₀₀ of 0.5 in MgSO₄. Aliquots of the library suspension containing 5×10⁴ pfu/ml (plaque forming unit per ml) were combined with 600 µl of XL1-Blue MRF' cells into sterile Falcon tubes that were incubated for 15 min at 37°C to allow the phages to attach to the cells. Then, 6.5 ml of NZY top agar, melted and cooled to 48°C, were added to each tube and the mixture was spread onto a freshly poured 140-mm NZY agar plate. The plates were let to settle for 10 min and then incubated at 37°C for 6–8 h. We did not allow the plaques to get larger than 1-2 mm. Each plate was overlaid with ~8–10 ml of SM buffer and stored at 4°C overnight (with gentle rocking). Bacteriophage suspension was recovered from each plate and pooled into a sterile polypropylene container. The plates were rinsed with an additional 2 ml of SM buffer and pooled. Chloroform was added (final concentration 5%), mixed and incubated for 15 min at room temperature. The cell debris were removed by centrifugation for 10 min at 2500 rpm. The supernatant was recovered and transferred into a sterile polypropylene container and 0.3% (vol/vol) of chloroform was added. The suspension was stored at 4°C until used. We can see all the process in Figura/Figure 3.3.



Figura/Figure 3.3 Amplification process of λ ZapII phage library.

3.15.2 Titration and ratio of recombinant bacteriophages of the cDNA library

Material

✓ **0.2% (wt/vol) maltose**

✓ **0.5 M IPTG**

IPTG (isopropyl β -D-1-thiogalactopyranoside; Sigma-Aldrich, USA) 0.25 g

DMF (N, N-Dimethylformamide; Sigma-Aldrich, USA) 0.4 ml

✓ **10 mM MgSO₄**

MgSO₄ 4.92 g

Distilled water up to 20 ml

✓ **0.1 M X-Gal**

X-Gal (5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside; Thermo Fisher, USA) 0.59 g

Distilled water 5 ml

✓ **Luria-Bertani (LB) broth/agar**

✓ **NZYM Agar/top Agar**



✓ **Dilution buffer (SM buffer; Teknova, USA)**

Method

A single colony of *Escherichia coli* XL1-Blue MRF' grown overnight in LB agar supplemented with tetracycline (10 µg/ml; filtered Φ : 0.22 µm; Sigma-Aldrich, USA), was inoculated into 50 ml of LB broth supplemented with 10 mM MgSO₄ and 0.2% (wt/vol) maltose (filtered Φ : 0.22 µm). The suspension was incubated overnight at 30°C and 240 rpm. Afterwards, the cells were spun down at 3500 rpm for 10 min and then cells were suspended and adjusted to an OD₆₀₀ of 0.5 in MgSO₄. We prepare the experiment in duplicate in order to run phage titration and ratio of recombinant bacteriophages determination (Tabla/Table 3.11).

Tabla/Table 3.11 Phage display titration and ratio of recombinant bacteriophage determination: package ligation conditions.

Type of Assay	Petri dishes diameter	<i>E.coli</i> (OD ₆₀₀ 0.5)	Phage				X-Gal	IPTG
			dilution in SM buffer (1/250000)	SM buffer	Top agar			
Titration						--	--	
Ratio of recombinant	90 mm	200 µl	2/5/10 µl	100 µl	3 ml	30 µl	30 µl	

Aliquots of 2, 5 or 10 µl of the library suspension diluted 1:250000 in SM buffer were combined with 200 µl of XL1-Blue MRF' cells and 100 µl of SM buffer into sterile Falcon tubes that were incubated for 15 min at 37°C to allow the phages to attach to the cells (Tabla/Table 3.11). We added 30 µl of X-Gal and 30 µl of IPTG in those Falcon tubes where we determined the ratio of recombinants. Then, 3 ml of NZY top agar, melted and cooled to 48°C were added to each tube and the mixture was spread onto a freshly poured 90-mm NZY agar plate. After 10 min, the plates were incubated at 37°C. After the incubation, the plaques were counted, the titer was established in plaque forming unit (pfu) per milliliter and the ratio of phages recombinant in percentage. The assay is schematized in Figura/Figure 3.5.

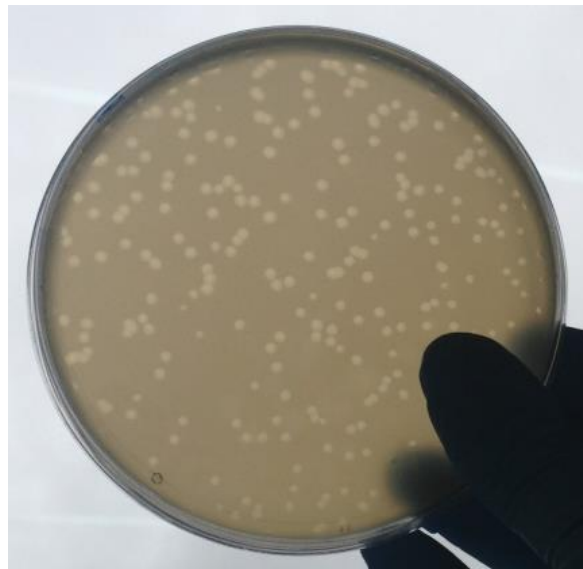


Phage titer was established using the following formula:

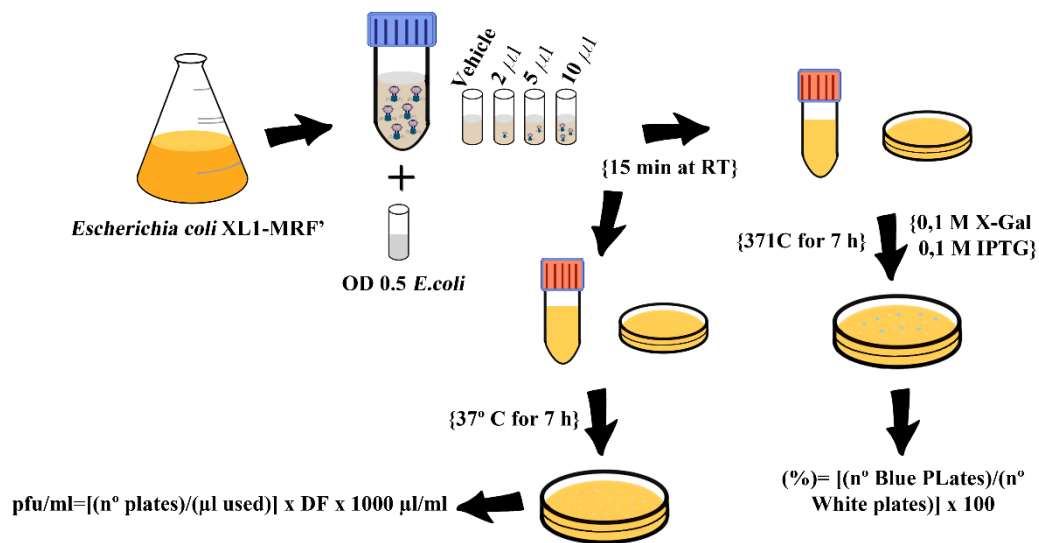
$$\frac{pfu}{ml} = \frac{n^{\circ} \text{ plates}}{\mu l \text{ used}} \times \text{dilution factor (DF)} \times 1000 \mu l/ml$$

Ratio of recombinants was determined using the following formula:

$$(\%) = \frac{n^{\circ} \text{ blue plaques}}{n^{\circ} \text{ white plaques}} \times 100$$



Figura/Figure 3.4 Lysis plaques of λ ZapII phage in LB agar.



Figura/Figure 3.5 Titration and ratio of recombinant phages of the cDNA library.

3.15.3 Library screening using CAGTA

Material

- ✓ **0.5% (vol/vol) Tween 20 in TBS**

Tween 20 (Sigma-Aldrich, USA) 0.05 ml

TBS (tris buffered saline) 100 ml

- ✓ **1% (wt/vol) BSA in TBS**

BSA (bovine serum albumin; Sigma-Aldrich, USA) 1 g

TBS (tris buffered saline) 100 ml

- ✓ **Chloroform (Sigma-Aldrich, USA)**
- ✓ **Luria-Bertani (LB) broth/agar**
- ✓ **NZYM agar/top agar (Teknova, USA)**
- ✓ **Primary antibody**

CAGTA purified from immune serum 1:60 in TBS-BSA

CAGTA-enr serum fraction 1:60 in TBS-BSA

- ✓ **Secondary antibodies**

Anti-rabbit IgG POX (Sigma-Aldrich, USA) 1:200 in BSA



Anti-human IgG POX (Sigma-Aldrich, USA) 1:200 in BSA

✓ **Resuspension buffer**

50 mM Tris-HCl (tris(hydroxymethyl)aminomethane; Panreac, Spain)	0.78 g
10 mM EDTA (ethylenediaminetetraacetic acid; Sigma-Aldrich, USA)	0.29 g
Distilled water	up to 1 l
pH adjusted to 8	

✓ **TBS buffer**

50 mM Tris-HCl pH 7.9 (Sigma-Aldrich, USA)	6.05 g
150 mM NaCl (Panreac, Spain)	8.76
Distilled water	up to 1l

Method

Previously to the screening, sample containing antibodies were incubated with *E. coli* lysate in order to eliminate cross-reactive antibodies and prevent false reactions. We followed the protocol described by Sambrook and colleagues (Sambrook & Russel, 2001).

E. coli was grown overnight at 30°C and 240 rpm in 100 ml of LB broth. The cells were harvested by centrifugation at 5500 rpm for 10 min at 4°C. The pellet was suspended in 3 ml of suspension buffer, frozen-thawed 3 times and sonicated at full power for six periods of 20 seconds. The extract was centrifuged at 12000 rpm for 10 min and the supernatant was stored at 4°C until used.

Two volumes of IgG solution, previously diluted 1:10 in TBA-BSA were mixed with one volume of *E. coli* lysate and incubated at RT for 4 h.

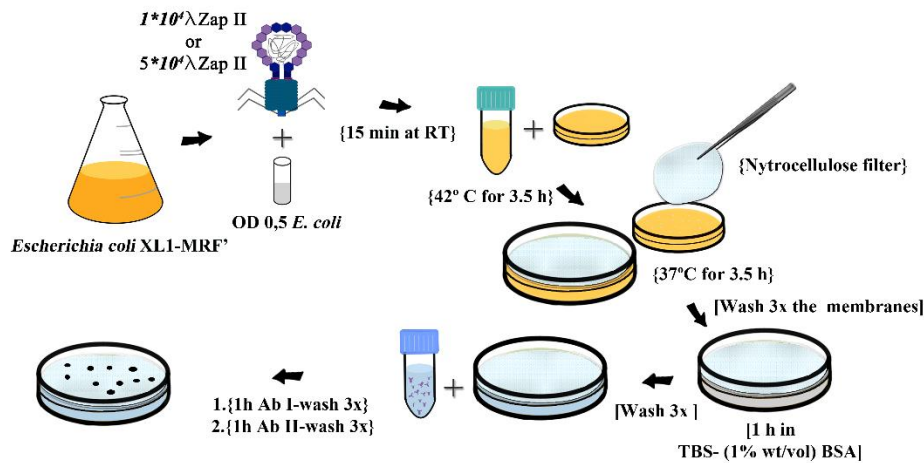
Antibody screening of cDNA libraries is facilitated by lambda ZapII phage expression vector that produce fusion proteins which can be detected on membrane filters by CAGTA. The screening was realized following the protocol of Sambrook and colleagues (Sambrook & Russel, 2001). A total of 30.000 plaques were analyzed using 140 mm diameter Petri dishes.

In order to do that, we mixed the components as schematized in Tabla/Table 3.12.

**Tabla/Table 3.12** Phage display screening conditions: package ligation preparation.

Petri dishes diameter	<i>E.coli</i> (OD₆₀₀ 0.5)	Phage plaques	SM buffer	Top agar
90 mm	200 µl	1x10 ⁴ pfu	100 µl	3 ml
140 mm	600 µl	5x10 ⁴ pfu	250 µl	6 ml

The *E. coli* preparation is described above in sections 3.15.1. The phage suspension with the bacteria was incubated at 37°C for 15 min to allow the phage to attach to the cells. Then, NZY top agar, previously melted and cooled to 48°C, was added into each Falcon tube, mixed and plated immediately onto dry, pre-warmed NZY agar plates. After 10 min, the plates were incubated at 42°C for 3.5 h until little plaques, around 1-2 mm of diameter, appeared. At that time, a dry nitrocellulose filter, previously saturated for 30 min with 10 mM IPTG, was placed on the top of the agar plates. They were incubated at 42°C for an additional period of 3.5 h. The filters were removed and placed either into 5 or 10 ml of TBS-Tween 20 for 3 min (depending on the plate diameter). They were blocked using TBS-BSA for 1 h. The western blot protocol was carried out as explained in section 3.8.4. In order to develop the reaction, the membrane was incubated for 5 min with the Clarity™ Western ECL Substrate kit (Bio-Rad, USA). The positive clones were visualized using the Chemidoc Quantity One system (BioRad, USA). The scheme of the protocol is shown in Figura/Figure 3.6.



Figura/Figure 3.6 Antibody screening in λ Zap II.

Plaques corresponding to the positive clones were located in their original plates. They were picked and suspended in 500 μ l of SM buffer with 0.3% (vol/vol) of chloroform and stored at 4°C.

3.15.4 Polymerase chain reaction and primer design of positive clones

Material

- ✓ **Agarose (Pronadisa, Spain)**
- ✓ **TAE buffer (Tris-Acetate-EDTA; 50x)**

Trizma Base (Sigma-Aldrich, USA)	242 g
0.5 M EDTA (ethylenediaminetetraacetic acid; Sigma-Aldrich, USA)	100 ml
Acetic acid-glacial (Sigma-Aldrich, USA)	57.1 ml
Distilled water	up to 1 l

Method

Polymerase chain reaction was used in order to sequence our positive clones. T3 and T7 primers were previously designed by Agilent Technologies (USA). The remaining primers were designed with the aid of Integrated DNA Technologies (IDT, USA). Primer characteristics are specified in Tabla/Table 3.13.

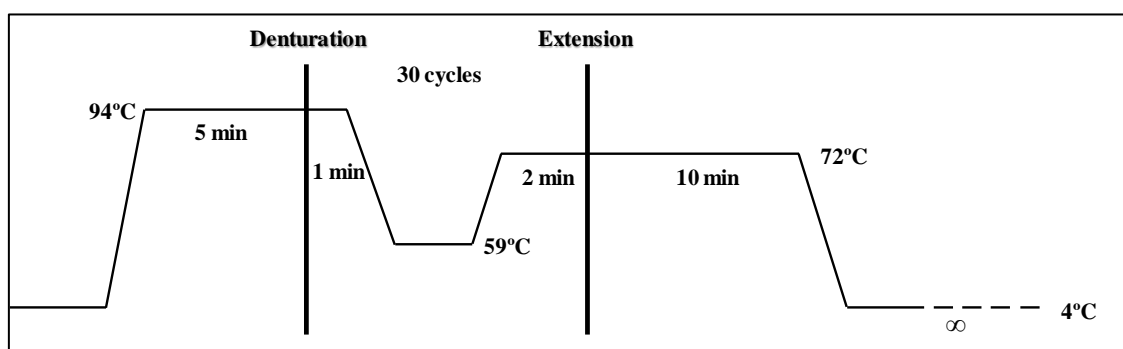
**Tabla/Table 3.13** Primers used in this study.

Primer	Sequence (5' → 3')	T _m	%GC	Size (pb)
T3	5' AATTAACCCTCACTAAAGGG	56°C	40	20
T7	5' GTAATACGACTCACTATAGGGC	56°C	45.45	22
F4a	5' AAAGATGTATACAGATGCGCCT	58°C	36.36	22
F4b	5'GGCTTGCTAACATCAAAGATGT	58°C	40.90	22
I4a	5'CTTCACTTTTCGGTACAAACACTG	61°C	43.46	23
I4b	5'GAACATGAAGTAACAGTGACAACAG	63°C	40	25
R1a	5'AAGATATACTCAAGTATGACGAACC	61°C	36	25
R1b	5'ATTAGCAGAAGAGAGTAAAGGTGAT	61°C	36	25
R2a	5'ATCTCTGCCACTGCTGAA	54°C	50	18
R2b	5'ACAAGCAAACGATCAAACAGAG	58°C	40.90	22

Amplifications were carried out in the thermocycler GeneAmp PCR System 9700 (Applied Biosystems, Thermo Fisher Scientific, USA). The reaction mix is detailed in Tabla/Table 3.14, and the scheme of PCR in Figura/Figure 3.7.

Tabla/Table 3.14 Mix reactions used in this study.

Reactives	Volume
BioMix (2X) (Bioline UK)	10 µl
Primer 1 (0.6 µM) - Forward	0.5 µl
Primer 2 (0.6 µM) - Reverse	0.5 µl
DNA template	1 pick
Milli-Q water	7 µl

**Figura/Figure 3.7** PCR cycle carried out in this study.



After each PCR, positive clones were corroborated by electrophoresis in 1.2% (wt/vol) agarose gels (Low Agarose, Pronadisa, Spain), with TAE buffer 1x for 60 min with 60V.

Amplicons were sequenced in the General Genomic Service for Sequencing and Genotyping Unit (SGIker, UPV-EHU).

The sequences were analyzed with BioEdit software (Biological Sequence Alignment Editor, Ibis Biosciences, USA) and their open reading frames were confirmed. The identification of each clone was carried out comparing our sequence with those registered in databases using the BLAST program (Basic Alignment Search Tool, <http://blast.ncbi.nlm.nih.gov/Blast>, USA).

3.16 Induction of damage by *Candida* Candidalysins

3.16.1 *Candida albicans* strains and growth conditions

Material

✓ YPD broth/agar

Peptone (Melford, UK)	20 g
Yeast extract (Oxoid, USA)	10 g
Glucose (VWR, USA)	20 g
Distilled water	up to 1l

For YPD agar, YPD broth was supplemented with 1.5% (wt/vol) agar (Melford, UK) prior to sterilization at 121°C for 15 min.

**Tabla/Table 3.15** *C. albicans* mutant strains used in this study.

Identity	Genotype	Reference
BWP17 + CIp30	<i>ura3::λimm434/ura3::λimm434</i> <i>iro1::λimm434/iro1::λimm434</i> <i>his1::hisG/his1::hisG</i> <i>arg4::hisG/arg4::hisG</i> <i>RPS1/rps1::(URA3-HIS1-ARG4)</i>	(Moyes et al., 2016)
<i>Kex1 Δ/Δ</i>	<i>ura3::λimm434/ura3::λimm434</i> <i>iro1::λimm434/iro1::λimm434</i> <i>his1::hisG/his1::hisG</i> <i>arg4::hisG/arg4::hisG</i> <i>kex1::HIS1/kex1::ARG4</i> <i>RPS1/rps1::URA3</i>	(Moyes et al., 2016)
<i>kex1Δ/Δ+KEX1</i>	<i>ura3::λimm434/ura3::λimm434</i> <i>iro1::λimm434/iro1::λimm434</i> <i>his1::hisG/his1::hisG</i> <i>arg4::hisG/arg4::hisG</i> <i>kex1::HIS1/kex1::ARG4</i> <i>RPS1/rps1::(URA3-KEX1)</i>	(Richardson et al., 2018)

C. albicans cells (Tabla/Table 3.15) were routinely grown on YPD agar at 37°C for 48 h.

3.16.2 Cell line and growth conditions

Material

- ✓ **DMEM medium supplemented with 1% (vol/vol) PS and 15% (vol/vol) FBS**

DMEM (Dulbecco's Modified Eagle's Medium; Sigma-Aldrich, USA)	425 ml
Heat-inactivated FBS (Fetal bovine serum; Gibco, USA)	75 ml
PS (Penicillin-Streptomycin; Sigma-Aldrich, USA)	0.5 ml

- ✓ **Sterile PSB (Sigma-Aldrich, USA)**
- ✓ **Trypsin solution (0.04 % with no EDTA; Sigma-Aldrich, USA)**

Method

All the experiments were performed using TR146 human oral epithelial cell line from the European Collection of Authenticated Cell Cultures (ECACC, UK), and it was originally isolated from neck metastasis (Rupniak et al., 1985). TR146 cells were grown to confluence in 25 ml of Dulbecco's Modified Eagle's Medium supplemented



with 15% (vol/vol) of fetal bovine serum and 1% (vol/vol) of penicillin-streptomycin in T175 flasks (Biofil, China) at 37°C and 5% CO₂.

In order to grow up cells from the stock, they were quickly thawed in a 37°C water bath. Nine milliliters of cold Dulbecco's Modified Eagle's medium supplemented with 10% (vol/vol) FBS and 1% (vol/vol) of penicillin-streptomycin was added to the cells. The solution was centrifuged for 5 min at 1200 rpm. The pellet was suspended in the remaining media in the Falcon and 10 ml of new media were added. Finally, the cells were transferred into a new T175 flask and other 15 ml of media were added. The cells were incubated at 37°C and 5% CO₂ for 24 h and then the media was changed.

TR146 cells can be stored in freezing medium. Trypsinized cells were collected and centrifuged at 1200 rpm for 5 min. The pellet was suspended in freezing medium consisting of 90% (vol/vol) heat inactivated fetal bovine serum and 10% (vol/vol) DMSO. One milliliter aliquots of cells were transferred to cryovials (at least 2x10⁶cell/ml). The cells were stored at -80°C in an isopropanol (Nalgene, USA) box for up to a week to slowly freeze down the cells. Finally, they were transferred to a liquid nitrogen tank for long-term storage.

Prior to the infection with *C. albicans* strains or incubation with Candidalysins, confluent cells were serum-starved overnight. Briefly, all medium was removed from the flask and the cells were washed with 10 ml of sterile PBS. The PBS was withdrawn and 4 ml of trypsin solution were added into the flask. After an incubation at 37°C and 5% CO₂ for 15 min, 8 ml of fresh media (pre-heated at 37°C) were added and trypsinized cells were counted on a BioRad Cell Counter (BioRad, USA). The cells were diluted at 5x10⁵ cell/ml and distributed in cell culture plates for further studies Table/Table 3.16.

**Tabla/Table 3.16** Cell seeding conditions.

Assays	Plate	Volume
Transcription factor DNA binding	6 cm (Cellstar, USA)	5 ml
Damage and Cytokines	24 well (Corning, USA)	500 μ l
Signalling proteins	12 well (Corning, USA)	1 ml

3.17 TransAm assay

Material

- ✓ *Candida* strains
- ✓ DMEM culture medium (Sigma-Aldrich, USA)
- ✓ Sterile PBS (Sigma-Aldrich, USA)
- ✓ YPD culture medium

Method

c-Fos DNA binding TransAm assay was performed using TransAM[®] AP-1 transcription factor ELISA kits (Active Motif[®], USA). This assay was used to study the transcription factors activated by MAPK signal transduction pathways. Briefly, all *C. albicans* strains were grown overnight at 30°C and 200 rpm. The following day, one milliliter of each culture was centrifuged and the pellet was washed twice with sterile PBS (Sigma-Aldrich, USA). The optical density of each culture diluted 1/100 in PBS was measured at 600 nm using a spectrophotometer (Perkin Elmer, USA) and the appropriate dilution required for the infection was determined using a previously established standard curve.

Five milliliters of 5×10^5 cell/ml were seeded into 6 cm dishes (Cellstar, USA), grown for 24 h until confluence and then serum-starved for 24 h prior to the infection. The epithelial cell lines were infected and incubated at 37°C and 5% CO₂ for 3 h with 1×10^8 cell/ml of different mutant strains of *Candida albicans* (Tabla/Table 3.15).

After that, the cells were scraped and assayed using a TransAM Transcription Factor ELISA kit according to the manufacturer's protocol. The absorbance measurements



at 492 nm were registered using the spectrophotometer Infinite® F50 (Tecan, Switzerland) and the software Magellon™–Data Analysis Software (Tecan, Switzerland).

3.18 Epithelial damage and cytokine production determination

Material

- ✓ Candidalysin peptides
- ✓ DMEM medium (Sigma-Aldrich, USA)
- ✓ Sterile PBS (Sigma-Aldrich, USA)

Method

Five hundred microliters of a TR146 cell suspension at a density of 5×10^5 cells/ml were grown to confluence and then serum-starved in serum-free DMEM prior to the incubation with peptides in 24 well plates. Each well was supplemented with Candidalysin (Table/Table 3.17) peptides to reach different final concentrations (10, 50 and 250 $\mu\text{g/ml}$; final volume 500 $\mu\text{l/well}$). The peptides were synthesized by Proteogenix (France) or Peptide Synthetics (UK). Peptide K and H stocks were diluted at 10 mg/ml in sterile water, and peptide E and A were diluted at 5 mg/ml in 10% of DMSO (Sigma-Aldrich, USA). Epithelial damage or cytokine production was determined after 24 h at 37°C and 5% CO₂. All the experiments were carried out in triplicate; the data were analyzed using the one-way ANOVA with all compared groups. *P* values < 0.05 were considered statistically significant. Sigma Plot software (version 12.5, Systat Software Inc., USA) was used for the analysis.

**Tabla/Table 3.17** Candidalysin peptides used in these experiments and their sequences.

Candidalysin origin	Amino acid Sequence
<i>Candida albicans</i> SC5314	SIIGIIMGILGNIPQVIQIIMSIVKAFKGNK
	SIIGIIMGILGNIPQVIQIIMSIVKAFKGNA
	SIIGIIMGILGNIPQVIQIIMSIVKAFKGNE
	SIIGIIMGILGNIPQVIQIIMSIVKAFKGNH
<i>Candida tropicalis</i> ATTC 750	ISFAGIVSSIINQLPSIIQIIGNIIKAGLVK
	ISFAGIVSSIINQLPSIIQIIGNIIKAGLVA
	ISFAGIVSSIINQLPSIIQIIGNIIKAGLVE
	ISFAGIVSSIINQLPSIIQIIGNIIKAGLVH
<i>Candida dubliniensis</i> CD36	SIIGILTAILNNVPQIINVITTIKSITGNK
	SIIGILTAILNNVPQIINVITTIKSITGNA
	SIIGILTAILNNVPQIINVITTIKSITGNE
	SIIGILTAILNNVPQIINVITTIKSITGNH
<i>Candida maltosa</i> Xu316	ASVGLISGIITNLPQLFQIISSILKSSGLVK
<i>Candida albicans</i> 529L	SFLSIITALLGNIPQIQMGIVKAFRGNK

3.18.1 Cell damage assay

Epithelial cell damage was determined after 24 h of incubation at 37°C and 5% CO₂ with different concentrations of Candidalysin by measuring lactate dehydrogenase (LDH) activity in the culture supernatant, as described previously by Moyes and colleagues (Moyes et al., 2010). Briefly, 40 µl of supernatant from each well were withdrawn and the assay was performed using the Kit Cyttox 96 Non-Radioactive Cytotoxicity provided by Promega (UK) according to the manufacturer's protocol. The assay quantitatively measures LDH (lactate dehydrogenase) release upon cell lysis. LDH released in culture supernatants is measured with a coupled enzymatic assay, in which tetrazolium salt (INT) is converted into a red formazan product. The amount of color is proportional to the number of lysed cells. A standard curve with concentrations ranging from 15 to 960mU/ml was generated using recombinant porcine LDH (Sigma-Aldrich, USA).



Measurements were made using the spectrophotometer Infinite® F50 (Tecan, Swizerland) at 492 nm and the software Magellon™–Data Analysis Software (Tecan, Swizerland).

3.18.2 Cytokines determination

The cytokines levels were quantified using the Luminex 200 Bioplex machine (BioRad, USA). After 24 h of incubation of TR146 cells with Candidalysin peptides, 240 µl of supernatant from each well were recollected and cytokine levels were quantified using a Proteome Profiler™ Human Cytokine Array Kit (Bio-Techne, UK) according to the manufacturer's protocol. The data were analyzed using the Bioplex Manager 6.1 software (BioRad, USA). The signal produced was proportional to the amount of analyte bound. The standard, the microparticles (IL-1 α , IL-1 β , IL-6, GM-CSF and G-CSF) and samples are all pipetted into 96 well plates (Corning, USA). We can see map plates in Tabla/Table 3.18.

Tabla/Table 3.18 Map plates used to carry out the Luminex Assays (a and b) to detect cytokines (IL-1 α , IL-1 β , IL-6, GM-CSF, G-CSF).

Luminex plate a	Standard curve											
	1	2	3	4	5	6	7	8	9	10	11	12
A	STD	STD	ALB 250K	ALB 50K	ALB 10K	ALB 250H	ALB 50H	529L 10	529L 250	MAL 50 K	MAL 10 K	VEHICLE
B	STD	STD	ALB 250K	ALB 50K	ALB 10K	ALB 250H	ALB 50H	529L 10	529L 250	MAL 50 K	MAL 10 K	VEHICLE
C	STD	STD	ALB 50K	ALB 10K	ALB 250H	ALB 50H	ALB 10H	529L 250	529L 50	MAL 10 K	MAL 250 K	VEHICLE
D	STD	STD	ALB 50K	ALB 10K	ALB 250H	ALB 50H	ALB 10H	529L 250	529L 50	MAL 10 K	MAL 250 K	VEHICLE
E	STD	STD	ALB 10K	ALB 250K	ALB 50H	ALB 10H	529L 250	529L 50	529L 10	MAL 250 K	MAL 50 K	VEHICLE
F	STD	STD	ALB 10K	ALB 250K	ALB 50H	ALB 10H	529L 250	529L 50	529L 10	MAL 250 K	MAL 50 K	VEHICLE
G	STD	STD	ALB 250K	ALB 50K	ALB 10H	ALB 250H	529L 50	529L 10	MAL 250 K	MAL 50 K	MAL 10 K	
H	STD	STD	ALB 250K	ALB 50K	ALB 10H	ALB 250H	529L 50	529L 10	MAL 250 K	MAL 50 K	MAL 10 K	

Luminex plate b	Standard curve											
	1	2	3	4	5	6	7	8	9	10	11	12
A	STD	STD	DUBL 250K	DUBL 50K	DUBL 10K	DUBL 250H	DUBL 50H	TROP 10K	TROP 250K	TROP 50H	TROP 10H	VEHICLE
B	STD	STD	DUBL 250K	DUBL 50K	DUBL 10K	DUBL 250H	DUBL 50H	TROP 10K	TROP 250K	TROP 50H	TROP 10H	VEHICLE
C	STD	STD	DUBL 50K	DUBL 10K	DUBL 250H	DUBL 50H	DUBL 10H	TROP 250K	TROP 50K	TROP 10H	TROP 250H	VEHICLE
D	STD	STD	DUBL 50K	DUBL 10K	DUBL 250H	DUBL 50H	DUBL 10H	TROP 250K	TROP 50K	TROP 10H	TROP 250H	VEHICLE
E	STD	STD	DUBL 10K	DUBL 250K	DUBL 50H	DUBL 10H	TROP 250K	TROP 50K	TROP 10K	TROP 250H	TROP 50H	VEHICLE
F	STD	STD	DUBL 10K	DUBL 250K	DUBL 50H	DUBL 10H	TROP 250K	TROP 50K	TROP 10K	TROP 250H	TROP 50H	VEHICLE
G	STD	STD	DUBL250K	DUBL 50K	DUBL 10H	DUBL 250H	TROP 50K	TROP 10K	TROP 250H	TROP 50H	TROP 10H	
H	STD	STD	DUBL 250K	DUBL 50K	DUBL 10H	DUBL 250H	TROP 50K	TROP 10K	TROP 250H	TROP 50H	TROP 10H	

*The first two columns (1 and 2) represent the standard curve. The columns 3-11 represent the samples: *C. albicans* (ALB), *C. albicans* 529L (529L), *C. maltosa* (MAL), *C. dubliniensis* (DUBL) and *C. tropicalis* (TROP); peptide concentration (250, 50 and 10 $\mu\text{g/ml}$) and the Candidalysin peptide represented by the last letter of its amino acid sequence: K and H. The vehicles were added in the last column (12). In each plate there were added all three replicates. Each assay is represented by one color: experiment 1 is purple, experiment 2 is pink and experiment 3 is blue. Each sample (including the standard curve) is assayed in duplicate.



3.19 Protein expression

Material

- ✓ **Candidalysin peptides**
- ✓ **DMEM culture medium (Sigma-Aldrich, USA)**
- ✓ **Filtered PBS (Sigma-Aldrich, USA)**
- ✓ **Phosphatase inhibitors (Perbio Science, Belgium)**
- ✓ **Protease inhibitors (Sigma-Aldrich, USA)**
- ✓ **RIPA (radioimmunoprecipitation assay) buffer**

1 mM EDTA (0.5 M stock; Sigma-Aldrich, USA)	2 ml
150 mM NaCl (Sigma-Aldrich, USA)	8.76 g
0.1% (wt/vol) SDS (Sigma-Aldrich, USA)	1 g
1% (wt/vol) Sodium deoxycholate (Sigma-Aldrich, USA)	10 g
50 mM Tris-HCl pH 7.4 (VWR, USA)	50 ml
1% (vol/vol) Triton X-100 (Sigma-Aldrich, USA)	10 ml
Distilled water	up to 1 l

Method

One milliliter of TR146 cells was seeded at a density of 5×10^5 cell/ml into 12 well plates. The cell were grown to confluence and then serum-starved in serum-free DMEM, prior to the incubation with the peptides. Each well was supplemented with Candidalysin peptides to reach different final concentrations (10, 50 and 250 μ g/ml, Tabla/Table 3.17) and plates were incubated at 37°C and 5% CO₂ for 2 h. Then, all plates were placed in ice in order to stop the peptides activity. Total cell lysates were collected by removing the supernatant and washing 2 times with 250 μ l of ice-cold filtered PBS (Thermo Fisher Scientific, USA). TR146 cells in each well were treated with 120 μ l of modified RIPA buffer containing a cocktail of proteases (1/100, Sigma-Aldrich, USA) and phosphatases (1/100, Perbio Science, Belgium) inhibitors. Cells were scraped and the samples were recollected in Eppendorf tubes and incubated in ice for 30 min. The suspension was centrifuged at



13000 rpm and 4°C for 10 min. The supernatant was collected and stored at -20°C until used.

The protein concentration was estimated using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, USA), following manufacturer's instructions.

3.19.1 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Material

✓ 10% (wt/vol) APS

APS 98% (ammonium persulfate; Sigma-Aldrich, USA)	0.1 g
Milli-Q water	1 ml

APS is prepared shortly prior to use

✓ Resolving gel SDS (sodium dodecyl sulfate) – PAGE (12%)

Protoflow gel solution (National Diagnostic, USA)	10 ml
Protoflow resolving buffer (National Diagnostic, USA)	6.5 ml
Distilled water	8.22 ml
APS	250 µl
Temed (N.N.N'.N'-Tetramethylethylenediamine; Sigma-Aldrich, USA)	25 µl

✓ Running buffer

Tris-Glycine-SDS Buffer 10X (Sigma-Aldrich, USA)	100 ml
Milli-Q Water	900 ml

✓ Stacking gel SDS-PAGE

Protoflow gel solution (National Diagnostic, USA)	1.56 ml
Protoflow Stacking Buffer (National Diagnostic, USA)	3 ml
Distilled water	7.32 ml
APS	120 µl
Temed	12 µl



Method

After preparing SDS-gels, 15 μ l of protein samples were mixed with 1.3 μ l of 1mM DTT (Sigma-Aldrich, USA) and 11.3 μ l of Laemmli Buffer 10X (Alfa Aesar, USA). Twenty micrograms of protein were run per lane. The samples were heated at 90°C during 5 min and loaded into their corresponding pockets next to 3 μ l of a pre-stained protein Ladder (Invitrogen, USA). Electrophoresis was run at 90 mV for 2 h using Mini-PROTEAN® Tetra Vertical Electrophoresis Cell (BioRad, USA).

3.19.2 Protein electrotransfer

Material

✓ **Transfer Buffer (10X)**

Tris Base (Sigma-Aldrich, USA)	30 g
Glycerol (Sigma-Aldrich, USA)	11.26 g
Milli-Q water	up to 1 l

✓ **Transfer Buffer (1X)**

Transfer Buffer 10 X	100 ml
Methanol (VWR Chemicals, USA)	100 ml
Milli-Q Water	800 ml

Method

Nitrocellulose membranes (BioRad, USA) and Whatman Chromatography paper (Life Technologies, USA) were cut with the same size of the gel and soaked in the transfer buffer for 3 min. The gel and the membranes were sandwiched between sponges and two chromatography papers (Whatman). The sandwiched cassette was placed into a tank, with the gel facing the cathode. The tank was filled with transfer buffer and closed. The transfer was carried out at 100 mV for 1 h.

3.19.3 Western blot

Material

- ✓ **0.1% (vol/vol) Tween 20 in PBS**



TBS 10X (Tris Buffered Saline; Severn Biotech. UK)	100 ml
Milli-Q Water	900 ml
Tween 20 (Sigma-Aldrich, USA)	1 ml
✓ 5% (wt/vol) BSA in TBS	
BSA (Fisher Scientific, USA)	2.5 g
TBS	50 ml
✓ 5% (wt/vol) milk in TBS	
Skim milk powder (Merck Millipore, Germany)	2.5 g
TBS	50 ml
✓ Primary Antibodies	
MKP1 Rabbit Ab (Cell Signaling Technology, USA) 1:1000 in TBS-BSA	
C-Fos Rabbit Ab (Cell Signaling Technology, USA) 1:1000 in TBS-milk	
Anti-Actin Antibody, clone C4, Mouse Ab (Merck Millipore, USA) 1:10000 in TBS-milk 5%	
✓ Secondary Antibodies	
Anti-IgG POX mouse (Jackson ImmuneResearch, USA) 1:20000 in TBS-milk	
Anti-IgG POX rabbit (Jackson ImmuneResearch, USA) 1:10000 in TBS-milk	

Method

WB analysis was carried out to visualize the target proteins. The membranes were placed into blocking solution at room temperature for 1 h to prevent unspecific binding of the detecting antibody to the membrane. They were subsequently incubated overnight with the primary antibody solutions at 4°C. In case of MKP1 expression study, the membranes were previously washed 3 times with TBS-Tween 20. The following day the membranes were washed 3 times with TBS-Tween 20 for 10 min and then incubated at room temperature for 1 h with the corresponding anti-IgG POX-conjugate antibody. After a second washing step, target proteins were detected by incubating membranes with 5 ml of the chemiluminescent HRP substrate (Merck Millipore, Germany) for 5 min. Finally, they were placed on X-ray film cassettes. The image was revealed using High Performance Chemiluminescence films (Gel Healthcare, USA) by exposing the film to the membrane. The films were developed using JPI Automatic X-Ray film processor (Model P-33, JPI Healthcare, USA).



The membranes were stripped with the re-blot plus strong solution (1x, Merck Millipore, Germany) at RT for 30 min and reassayed to reveal the α -actin loading control.

4 Results





4

4.1 Caracterización de los anticuerpos desarrollados en un modelo animal de candidemia en conejo infectado con *Candida albicans*

4.1.1 Reacción de la fracción de IgG totales por inmunofluorescencia indirecta (IFI) frente a *Candida albicans*

La reacción de la fracción de IgG totales (total-IgG) de los sueros de conejo infectado 16706 (título AM 1/160, anti-BI 1/640) y 16715 (título AM 1/160, anti-BI 1/320) se verificó por inmunofluorescencia indirecta (IFI) frente a *Candida albicans*. La fracción total IgG reaccionó tanto con blastosporas como con los tubos germinales (Figura/Figure 4.1). En dicha figura se puede observar que a mayor título de anticuerpos específicos frente a *C. albicans* era necesaria una menor concentración de IgG para evidenciar la reacción, probablemente debido a la mayor representatividad de los anticuerpos anti-*Candida* entre las IgG totales. Así, el suero 16715 mostró reconocimiento a concentraciones de IgG $\geq 50 \mu\text{g/ml}$.

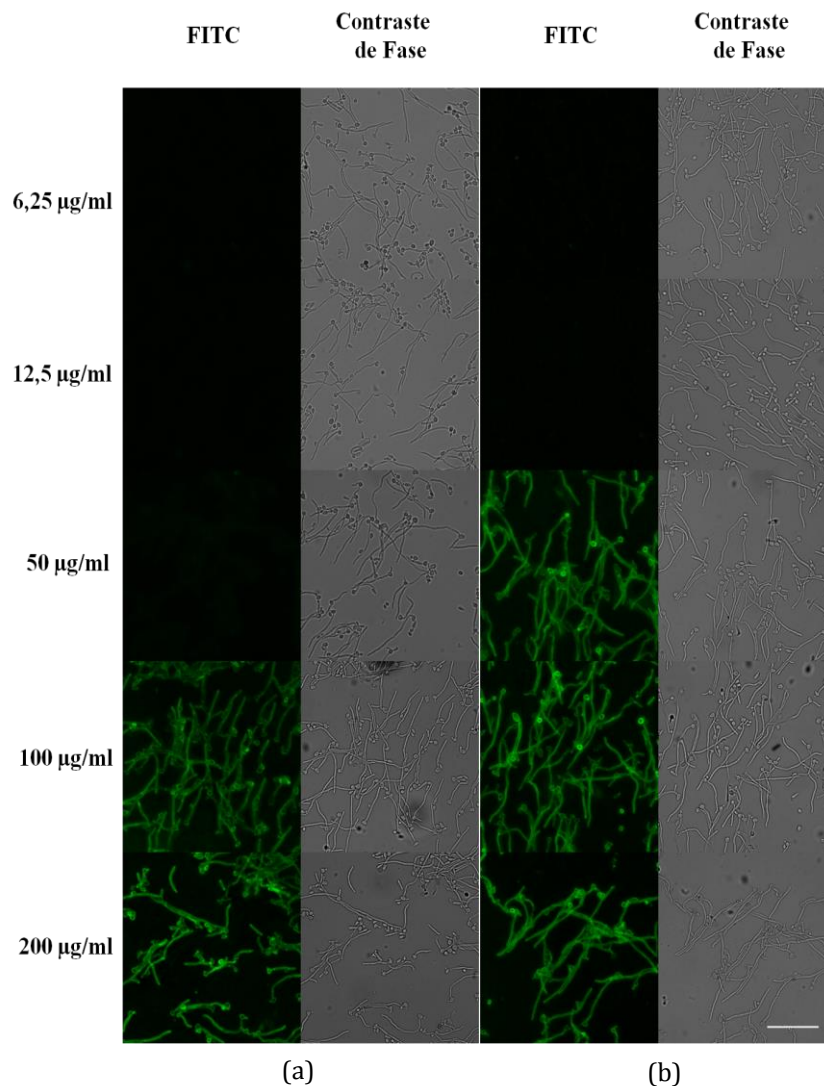
4.1.2 Caracterización de los CAGTA mediante ELISA

Mediante estudios de inmunoproteómica, en nuestro laboratorio se han identificado algunos antígenos reconocidos por los CAGTA (Sáez-Rosón et al., 2014). Estos antígenos incluyen proteínas que se han producido posteriormente en nuestro laboratorio de forma recombinante en *Escherichia coli*, entre las que se encuentran 14-3-3, Adh1, Als3-N, Eno-1, Met-6 y Hwp1-N. Con el fin de determinar la respuesta de anticuerpos frente a estos antígenos en el modelo animal y su evolución a lo largo de la infección, en primer lugar, se obtuvieron estas proteínas recombinantes para su posterior utilización en ensayos de ELISA.



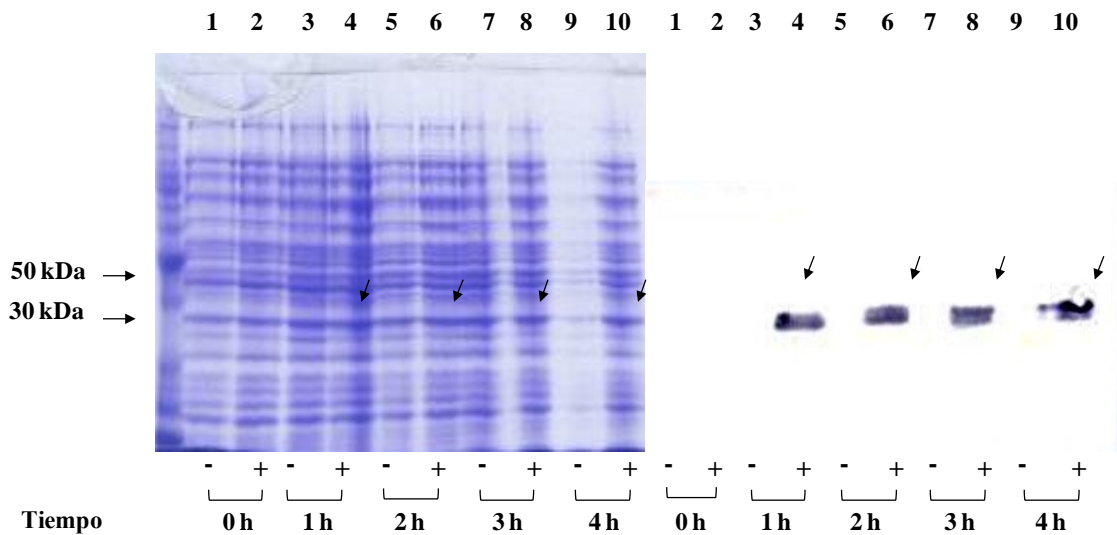
4.1.2.1 Producción de Hyphal Wall Protein (Hwp1-N)

Las cepas de *E. coli* transformadas con los plásmidos codificantes de las diferentes proteínas, construidas previamente por colaboradores de nuestro grupo de investigación, se cultivaron para obtener dichas proteínas recombinantes y su posterior purificación. Como ejemplo se muestra el proceso de producción y purificación de Hwp1-N siguiendo el protocolo descrito por Laín y colaboradores (Laín et al., 2007a) (Figura/Figure 4.2 y Figura/Figure 4.3).



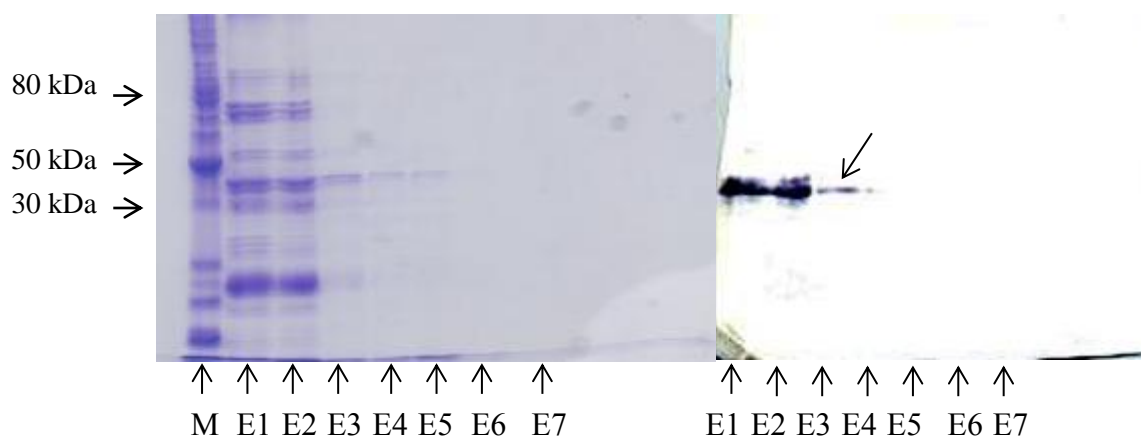
Figura/Figure 4.1 Reacción de IgG totales de los sueros de conejo 16706 (a) y 16715 (b) con tubos germinales de *C. albicans*. Inmunofluorescencia indirecta de la reactividad. Concentración de la fracción total de IgG (6,25-200 µg/ml). En cada caso se presentan parejas de imágenes: a la izquierda microscopía de fluorescencia con el anticuerpo secundario conjugado a FITC, y a la derecha su correspondiente imagen por microscopía óptica de contraste de fases. La barra representa 50 µm de longitud.





Figura/Figure 4.2 Expresión de la proteína Hwp1-N. Extractos proteicos, separados por SDS-PAGE, de los cultivos de *E. coli* Rosetta™ (DE3) conteniendo el plásmido recombinante para Hwp1-N, a lo largo del tiempo de incubación (0, 1, 2, 3 y 4 horas) a 37°C. El panel izquierdo muestra el gel teñido con azul Coomassie y el derecho la inmunodetección con el anticuerpo monoclonal anti-HSV Tag. Calles 2, 4, 6, 8 y 10: extractos de células inducidas con IPTG; Calles 1, 3, 5, 7, 9: extractos de células sin inducir.

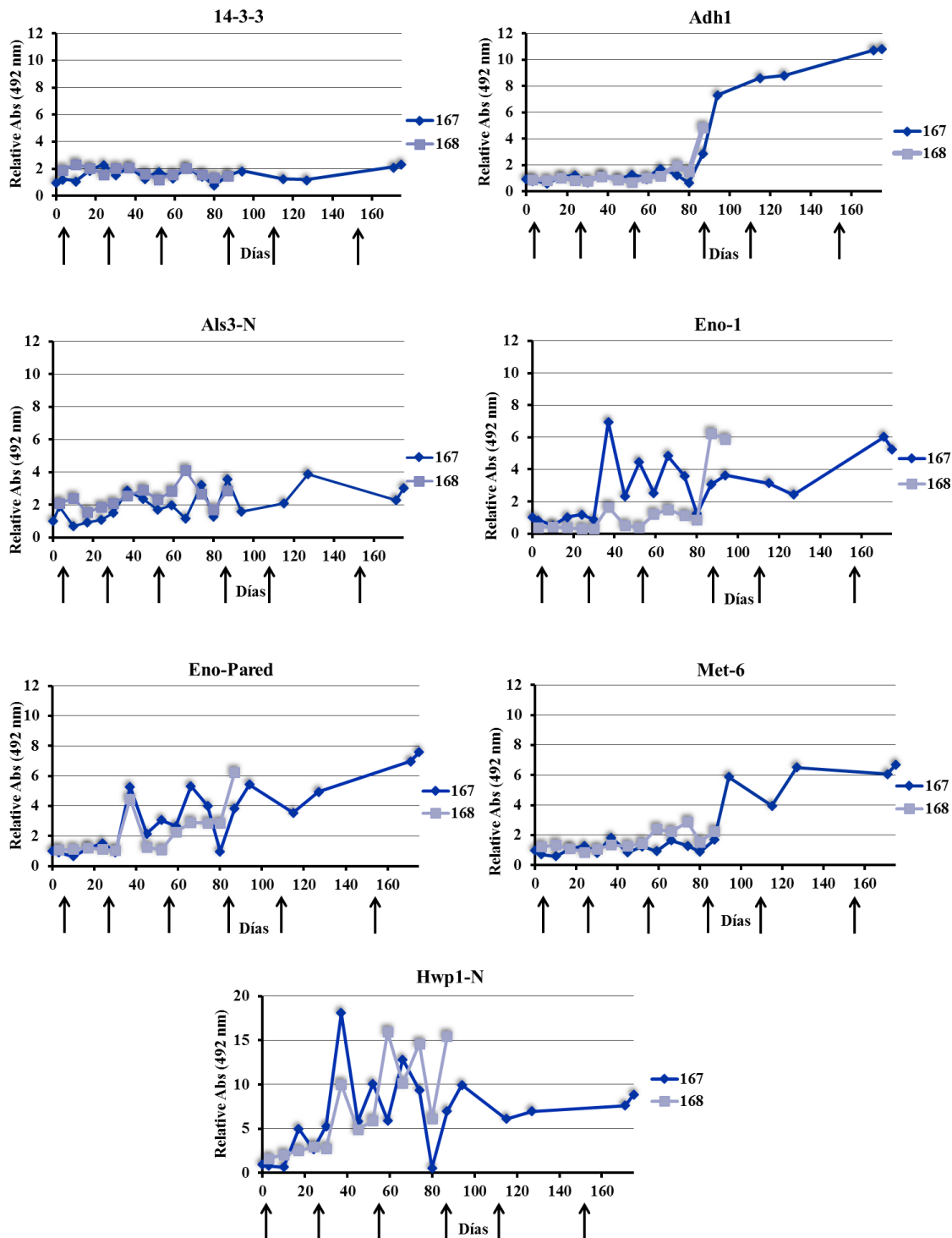
Una vez inducida la proteína (Figura/Figure 4.2), se purificó a partir de los sobrenadantes de cultivo mediante cromatografía de afinidad a níquel posibilitada por la presencia de una cola de histidinas. En cada purificación, se recogieron 7-8 eluidos de unos 3 ml cada uno y se verificó la presencia y pureza de la proteína por electroforesis SDS-PAGE seguida de una tinción con azul Coomassie y un análisis Western Blotting con el anticuerpo monoclonal antiHSV·Tag®, comprobando de esta forma en que fracción del eluido se encontraba la proteína de interés (Figura/Figure 4.3). En el caso de las calles E1 y E2 representadas en esta figura, los eluidos se volvieron a pasar por una columna cromatográfica, obteniendo de esta forma una fracción más pura de la proteína.



Figura/Figure 4.3 Eluidos recogidos en el proceso de purificación de la proteína recombinante Hwp1-N por cromatografía de afinidad a níquel. El panel izquierdo muestra el gel teñido con azul Coomassie y el derecho la inmunodetección con el anticuerpo monoclonal anti-HSV-Tag. Calles: M es el marcador de tamaño molecular; E1, E2, E3, E4, E5, E6 y E7: distintas fracciones de eluidos recogidas.

4.1.2.2 Evolución de los títulos de los anticuerpos frente a las proteínas recombinantes

Se estudió por ELISA la capacidad de reconocimiento de los anticuerpos desarrollados en el modelo animal de candidiasis frente a distintos antígenos de *C. albicans* seleccionados en trabajos previos de nuestro grupo. Como se puede observar en la Figura/Figure 4.4, la evolución de los títulos de anticuerpos IgG específicos para las diferentes proteínas en los dos conejos del ensayo fue muy similar. Todos los sueros mostraron reacción con los antígenos seleccionados, obteniendo los valores más altos frente a la proteína recombinante Hwp1-N, seguida por Adh1, Met-6, y Eno-1, así como con la enolasa purificada a partir de un extracto de pared de tubos germinales de *C. albicans*. En el caso de los anticuerpos anti-14-3-3 y anti-Als3-N los perfiles de ambos conejos fueron bajos y mantenidos. La aparición de anticuerpos con distintas especificidades siguió un patrón diferente para las proteínas ensayadas. Los anticuerpos contra Hwp1-N inician su elevación tras la primera inoculación con blastosporas vivas de *C. albicans*, alcanzando valores máximos tras la segunda inoculación. La reacción con Eno-1 y Eno-pared alcanza valores máximos tras la segunda inoculación y consecutivas. En contraste, los anticuerpos frente a Adh1 y Met-6 requirieron cuatro tandas de inoculación antes de elevar sus títulos.



Figura/Figure 4.4 Evolución de los títulos de anticuerpos (absorbancia relativa) en el modelo animal de candidiasis invasora (conejos 167 y 168) frente a las proteínas de *C. albicans* 14-3-3, Adh1, Als3-N, Eno-1, Eno-Pared, Met-6 y Hwp1-N. Las flechas se corresponden a los días de inoculación intravenosa con 2×10^6 blastosporas viables de *C. albicans* (0, 28, 56, 84, 112 y 154 días).



4.2 Selección de antígenos reconocidos por CAGTA mediante rastreo de una genoteca de *Candida albicans* en el fago λ ZapII

De forma complementaria, se intentó completar la lista de antígenos reconocidos por los CAGTA mediante el cribado de una genoteca de cDNA de micelios de *C. albicans* en el fago λ ZapII, cedida por el Dr. William Fonzi.

En primer lugar, se determinó el título de la genoteca, que fue del orden de $6,95 \cdot 10^9$ pfu/ml con un 97% de clones recombinantes y se amplificó para posteriores experimentos alcanzando un título de $1,97 \cdot 10^{10}$ pfu/ml.

4.2.1 Cribado de la genoteca de *Candida albicans* en λ ZapII

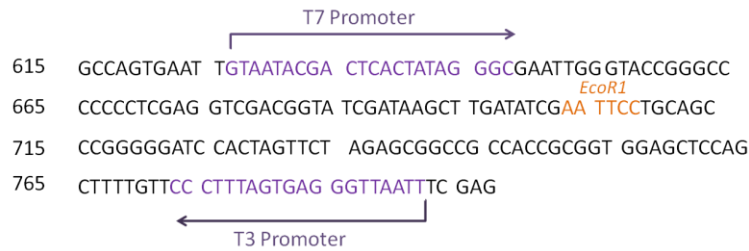
El cribado inicial para la selección de clones productores de las distintas proteínas reconocidas por los CAGTA, se efectuó con la fracción CAGTA-enr procedente del conejo 167.

De este modo se analizaron más de $1 \cdot 10^6$ placas de lisis, entre los que se seleccionaron 59 clones. Se repitió el ensayo de reacción con la fracción CAGTA-enr y se procedió a la caracterización de los insertos correspondientes. La amplificación con los iniciadores T3 y T7 que flanquean el lugar de inserción en el fago λ ZapII (Figura/Figure 4.5) permitió determinar el tamaño de los insertos en los fagos seleccionados, así como comprobar si estos se encontraban en fase para la expresión de la correspondiente proteína de fusión con β -galactosidasa.

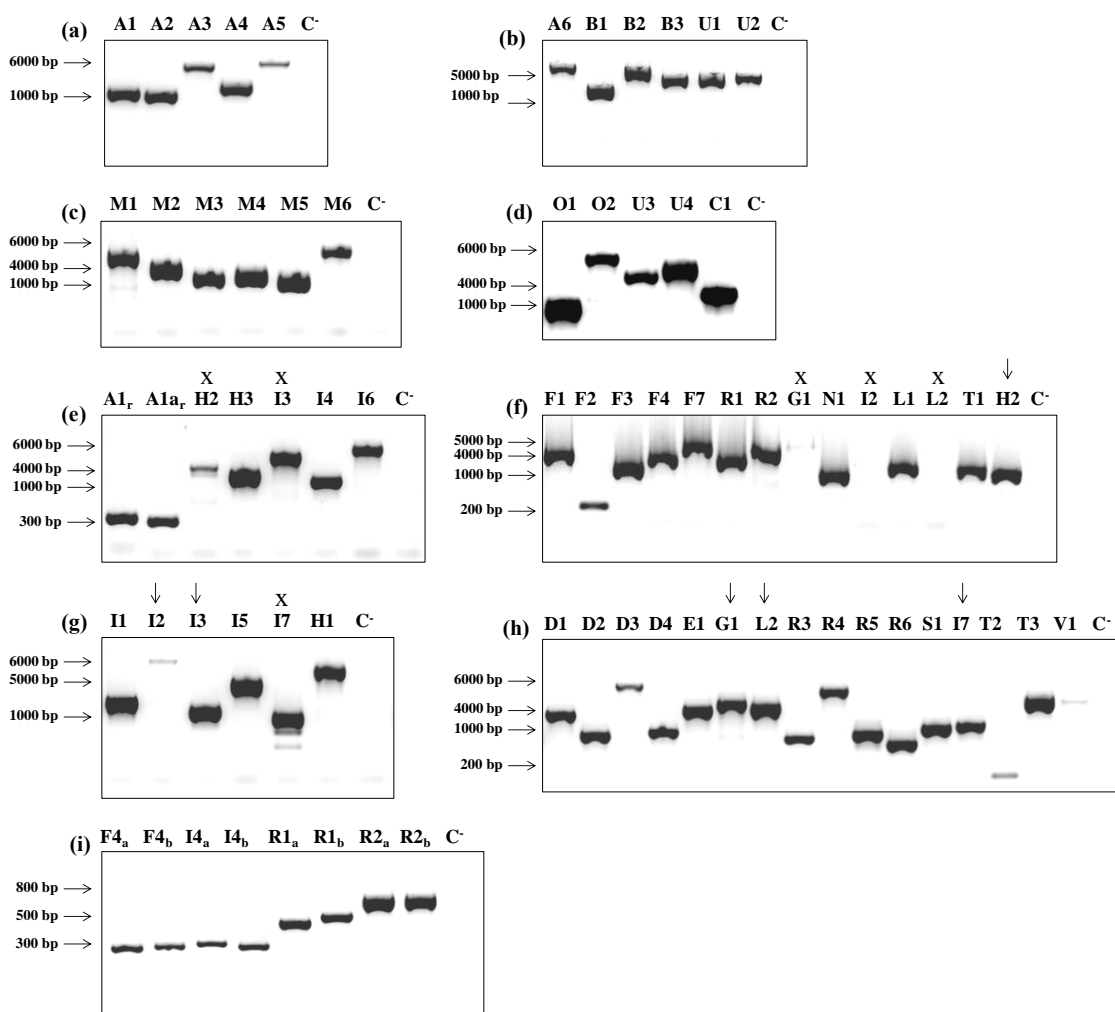
En la Figura/Figure 4.6 se muestra el tamaño de los insertos amplificados por PCR de los 59 clones aislados y denominados en función de la placa de origen. En el caso de los insertos A1, F4, I4, R1 y R2 se diseñaron unos cebadores más cercanos al punto de clonación *EcoRI*, con la herramienta *online* de Integrated DNA Technologies (IDT, EEUU), facilitando así su posterior secuenciación ya que con la amplificación T7-T3 no se consiguió llegar al punto de inserción y por tanto no se podía establecer su pauta de lectura (Figura/Figure 4.5). Cuando la calidad de la amplificación por PCR no mostró una sola banda o no permitía su secuenciación, se repitió la clonación para obtener un clon puro. En la Figura/Figure 4.6 se muestran algunas calles marcadas con una X, que se



corresponde a los clones G1, H2, I2, I3, I7, L2, para los que hubo que repetir el citado proceso de clonación.



Figura/Figure 4.5 Secuencia de nucleótidos de la zona de clonación del fago λ ZapII. En morado se indican las regiones T7 y T3 para la amplificación de los insertos clonados en el fago. En naranja se indica el lugar de corte con la enzima *EcoRI*.



Figura/Figure 4.6 PCR de los 59 clones seleccionados tras el rastreo inicial de la genoteca de *C. albicans* en el fago λ ZapII, amplificados con los cebadores T3 y T7. Las calles marcadas con una X corresponden a los amplicones de fagos para los que hubo que repetir la clonación (clones definitivos marcados con una flecha). Los amplicones indicados con un subíndice (a, b o r) corresponden a la amplificación con una pareja de cebadores diferentes (panel e: A1_r, A1_a_r; panel i: F4_a, F4_b, I4_a, I4_b, R1_a, R1_b, R2_a, R2_b). C: control negativo de la amplificación.

Se estudiaron las secuencias de los cDNA clonados en el lugar *EcoRI* y se seleccionaron aquellas que estaban en la orientación correcta y con la pauta de lectura en fase. De este



modo, se seleccionaron 5 clones positivos de un total de $1 \cdot 10^6$ analizados, los denominados: A2, D1, H1, N1 y R1.

Todas las secuencias de nucleótidos de los 5 clones mostraron una alta homología con secuencias del genoma de *C. albicans* registradas en las bases de datos de este hongo. Las Figura/Figure 4.7-Figura/Figure 4.11 muestran las alineaciones de las secuencias de los insertos de los clones seleccionados y la homología con las correspondientes secuencias genómicas registradas de *C. albicans*.



Candida albicans SC5314 chromosome R sequence					Clon A2
Sequence ID: CP017630.1 Length: 2286237 Number of Matches: 1					
Range 1: 1344143 to 1344823 GenBank Graphics					
Score	Expect	Identities	Gaps	Strand	
1247 bits(675)	0.0	679/681(99%)	0/681(0%)	Plus/Minus	
Query 60	CTAATCAAACATCTACAATCATGGCTCCTTTAAATTAGCTACCGATTTACCAGAATGGA				119
Sbjct 1344823	CTAATCAAACATCTACAATCATGGCTCCTTTAAATTAGCTACCGATTTACCAGAATGGA				1344764
Query 120	AAAAATTGGAAGAACTTACAAATCCGTCCGGTGAGAAATTCAGTGTCAGAGATGCATTTCG				179
Sbjct 1344763	AAAAATTGGAAGAACTTACAAATCCGTCCGGTGAGAAATTCAGTGTCAGAGATGCATTTCG				1344704
Query 180	CCAAAGACCCAAAAAGATTTGAAGAGTTCTCCTGGATCTACAAAACTACGATGACTCCA				239
Sbjct 1344703	CCAAAGACCCAAAAAGATTTGAAGAGTTCTCCTGGATCTACAAAACTACGATGACTCCA				1344644
Query 240	AGATCTTATTGACTTCTCCAAGAAGTTGGTTAACAAAGAAATCCTTGATCAATTGGTTA				299
Sbjct 1344643	AAATCTTATTGACTTCTCCAAGAAGTTGGTTAACAAAGAAATCCTTGATCAATTGGTTA				1344584
Query 300	CTTTGGCTAAAGAAGCCGGTGTGAAAAATTGAGAGACGCCATGTTGCTGGTGACCACA				359
Sbjct 1344583	CTTTGGCTAAAGAAGCCGGTGTGAAAAATTGAGAGACGCCATGTTGCTGGTGACCACA				1344524
Query 360	TCAACACAACCGAAGATAGAGCTGTTTACCACGTTGCTTTAAGAAACCGTGCTTTGAGAA				419
Sbjct 1344523	TCAACACAACCGAAGATAGAGCTGTTTACCACGTTGCTTTAAGAAACCGTGCTTTGAGAA				1344464
Query 420	AGATGCCAGTTGATGGGAAAGATACCGCTCAAGAAGTTGACGATGCTTTGAAACACATGA				479
Sbjct 1344463	AGATGCCAGTTGATGGGAAAGATACCGCTCAAGAAGTTGACGATGCTTTGAAACACATGA				1344404
Query 480	AAGAATTCAGTGACTCAATTAGAGACGGTTCTGGACTGGTTACACCGGCAATCCATTA				539
Sbjct 1344403	AAGAATTCAGTGACTCAATTAGAGACGGTTCTGGACTGGTTACACTGGCAATCCATTA				1344344
Query 540	CTGATGTTGTTAACATTGGTATTGGTGGTTCTGATTTGGGTCCTGTTATGGTCACTGAAG				599
Sbjct 1344343	CTGATGTTGTTAACATTGGTATTGGTGGTTCTGATTTGGGTCCTGTTATGGTCACTGAAG				1344284
Query 600	CTTTGAAAGCTTACAGTAAACCAGGTTTGAATGTCCACTTTATTTCTAACATTGACGGTA				659
Sbjct 1344283	CTTTGAAAGCTTACAGTAAACCAGGTTTGAATGTCCACTTTATTTCTAACATTGACGGTA				1344224
Query 660	CTCATACTGCTGAAACTTTAAAAAAGTTGAACCCAGAACTACTTTGTTTTGATTGCTT				719
Sbjct 1344223	CTCATACTGCTGAAACTTTAAAAAAGTTGAACCCAGAACTACTTTGTTTTGATTGCTT				1344164
Query 720	CCAAAACATTCACACTACTGCTG	740			
Sbjct 1344163	CCAAAACATTCACACTACTGCTG	1344143			

Figura/Figure 4.7 Representación gráfica de la secuencia del clon A2 y comparación con la secuencia genómica de *C. albicans* que mostraba la mayor homología en las bases de datos consultadas.



Candida albicans hyphal wall protein 1 (HWP1) gene, complete cds
 Sequence ID: [U64206.1](#) Length: 2682 Number of Matches: 5 **Clon D1**

Range 1: 656 to 1294 [GenBank](#) [Graphics](#) ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
1181 bits(639)	0.0	639/639(100%)	0/639(0%)	Plus/Plus
Query 52	CCacaacaacaacaacaacaagagccttgatgattaccacaacaacaacaGcAGGAAGAA			111
Sbjct 656	CCACAACAACAACAACAAGAGCCTTGTGATTACCCACAACAACAACAGCAGGAAGAA			715
Query 112	CCTTGTGATTACCCACAACAACAACCACAAGAGCCATGTGACTATCCACAACAGCCACAA			171
Sbjct 716	CCTTGTGATTACCCACAACAACAACCACAAGAGCCATGTGACTATCCACAACAGCCACAA			775
Query 172	GAACCTTGTGACTACCCACAACAACCACAAGAACCTTGTGACTACCCACAACAACCACAA			231
Sbjct 776	GAACCTTGTGACTACCCACAACAACCACAAGAACCTTGTGACTACCCACAACAACCACAA			835
Query 232	GAACCTTGCACAATCCACCTCAACCTGATGTTCTTGTGACAATCCTCCTCAACCTGAT			291
Sbjct 836	GAACCTTGCACAATCCACCTCAACCTGATGTTCTTGTGACAATCCTCCTCAACCTGAT			895
Query 292	GTTCTTGTGACAATCCTCCTCAACCTGATATTCTTGTGACAATCCTCCTCAACCTGAT			351
Sbjct 896	GTTCTTGTGACAATCCTCCTCAACCTGATATTCTTGTGACAATCCTCCTCAACCTGAT			955
Query 352	ATTCTTGTGACAATCCTCCTCAACCTGATCAGCCTGATGACAATCCTCCTATTCCAAC			411
Sbjct 956	ATTCTTGTGACAATCCTCCTCAACCTGATCAGCCTGATGACAATCCTCCTATTCCAAC			1015
Query 412	ATTCCAACCGATTGGATTCCAATATTCCAACCTGATTGGATCCCAGATATTCCAGAAAAG			471
Sbjct 1016	ATTCCAACCGATTGGATTCCAATATTCCAACCTGATTGGATCCCAGATATTCCAGAAAAG			1075
Query 472	CCAACAACCTCCAGCTACTACTCCAACATTCTGCTACAACCTACTActtctgaatcatca			531
Sbjct 1076	CCAACAACCTCCAGCTACTACTCCAACATTCTGCTACAACCTACTACTTCTGAATCATCA			1135
Query 532	tcttcttcttcttcttcatcatcatctACTACTCCAAAACTTCTGCTTCAACTACACCT			591
Sbjct 1136	TCTTCTTCTTCTTCTTCTCATCATCATCTACTACTCCAAAACTTCTGCTTCAACTACACCT			1195
Query 592	GAATCTTCTGTTCCAGCTACCACTCCAACACTTCTGTTCCAACAACCTTCTTCAAGATCA			651
Sbjct 1196	GAATCTTCTGTTCCAGCTACCACTCCAACACTTCTGTTCCAACAACCTTCTTCAAGATCA			1255
Query 652	ACTACTCCAGCTACTAGCCCAGAAAGTTCTGTTCCAGTT 690			
Sbjct 1256	ACTACTCCAGCTACTAGCCCAGAAAGTTCTGTTCCAGTT 1294			

Figura/Figure 4.8 Representación gráfica de la secuencia del clon D1 y comparación con la secuencia genómica de *C. albicans* que mostraba la mayor homología en las bases de datos consultadas.



Candida albicans SC5314 chromosome 5 sequence
Sequence ID: [CP017627.1](#) Length: 1190845 Number of Matches: 1 **Clon H1**

Range 1: 24366 to 25096 [GenBank](#) [Graphics](#) [Next Match](#) [Previous Match](#)

Score	Expect	Identities	Gaps	Strand
1345 bits(728)	0.0	731/732(99%)	1/732(0%)	Plus/Minus
Query 53	GTCTTCTGGTGAAGAGTTGACTTCAAAAAGCTCCTGGTACCAACGAGCCATATGCTATTGT			112
Sbjct 25096	GTCTTCTGGTGAAGAGTTGACTTCAAAAAGCTCCTGGTACCAACGAGCCATATGCTATTGT			25037
Query 113	GTCTGGTGATTACAATCCAATTACAGTTTCAAGAGTTTTGCTGCTTATGCTAAATTGCC			172
Sbjct 25036	GTCTGGTGATTACAATCCAATTACAGTTTCAAGAGTTTTGCTGCTTATGCTAAATTGCC			24977
Query 173	AGGTACTATCACTCATGGAATGTACTCGTCTGCTTCGATCAGAGCATTGGTTGAAGAATG			232
Sbjct 24976	AGGTACTATCACTCATGGAATGTACTCGTCTGCTTCGATCAGAGCATTGGTTGAAGAATG			24917
Query 233	GGCTGCCAACAAATGTTGCCGCAAGAGTTAGAGCATTCAAGTGTGATTTTCGTTGGTATGGT			292
Sbjct 24916	GGCTGCCAACAAATGTTGCCGCAAGAGTTAGAGCATTCAAGTGTGATTTTCGTTGGTATGGT			24857
Query 293	ATTGCCAAATGATACTTTGCAAACCTACCATGGAACATGTTGGTATGATCAATGGACGTAA			352
Sbjct 24856	ATTGCCAAATGATACTTTGCAAACCTACCATGGAACATGTTGGTATGATCAATGGACGTAA			24797
Query 353	GATCATCAAGGTTGAAACTAGAAATGTTGAAACTGAGCTTCCAGTTTTGATTGGGGAAGC			412
Sbjct 24796	GATCATCAAGGTTGAAACTAGAAATGTTGAAACTGAGCTTCCAGTTTTGATTGGGGAAGC			24737
Query 413	TGAAATTTGAACAACCAACAACCACTATGTTTTCACTGGTCAAGGTTCTCAAGAACAAGG			472
Sbjct 24736	TGAAATTTGAACAACCAACAACCACTATGTTTTCACTGGTCAAGGTTCTCAAGAACAAGG			24677
Query 473	TATGGGTATGGAGTTGTACAACCTCTTCTGAGGTTGCTCGTGAAGTTTGGGACAAAGCTGA			532
Sbjct 24676	TATGGGTATGGAGTTGTACAACCTCTTCTGAGGTTGCTCGTGAAGTTTGGGACAAAGCTGA			24617
Query 533	CAGACACTTTGTCAATAACTATGGTTTTCTATTTTGGATATTGTTCAAAACAATCCAAA			592
Sbjct 24616	CAGACACTTTGTCAATAACTATGGTTTTCTATTTTGGATATTGTTCAAAACAATCCAAA			24557
Query 593	TGAATTGACTATCCACTTTGGCGGTGCCAAAGGTAGAGCTATTAGAGACAACATATTGG			652
Sbjct 24556	TGAATTGACTATCCACTTTGGCGGTGCCAAAGGTAGAGCTATTAGAGACAACATATTGG			24497
Query 653	TATGATGTTTGAAACTATTGGTGAAGATGGTCTTTGAAGTCGGAAAAGATTTTCAAGGA			712
Sbjct 24496	TATGATGTTTGAAACTATTGGTGAAGATGGTCTTTGAAGTCGGAAAAGATTTTCAAGGA			24437
Query 713	TATCGATGAGACTACTACTTCTTACACATTTGTTTACCAACTGGTTTGGTTATCAGCAA			772
Sbjct 24436	TATCGATGAGACTACTACTTCTTACACATTTGTTTACCAACTGGTTTGGTTATCAGCAA			24378
Query 773	CTCAATTCACCC	784		
Sbjct 24377	CTCAATTCACCC	24366		

Figura/Figure 4.9 Representación gráfica de la secuencia del clon H1 y comparación con la secuencia genómica de *C. albicans* que mostraba la mayor homología en las bases de datos consultadas.



Candida albicans SC5314 Phr1p (PHR1), partial mRNA					Clon N1
Sequence ID: XM_712140.2 Length: 1647 Number of Matches: 1					
Range 1: 681 to 891 GenBank Graphics			▼ Next Match ▲ Previous Match		
Score	Expect	Identities	Gaps	Strand	
379 bits(205)	2e-101	209/211(99%)	0/211(0%)	Plus/Plus	
Query 69	AGATGATCGTGCTGATTTCTTTGGTATCAATATGTATGAATGGTGGCAAATCAACTTT				128
Sbjct 681	AGATGATCGTGCTGATTTCTTTGGTATCAATATGTATGAATGGTGGCAAATCAACTTT				740
Query 129	CGAAACCTCAGGTTACAAGGACAGAAGTGAAGAAATCAAGAACTTGACTATCCCAGCCTT				188
Sbjct 741	CGAAACCTCAGGTTACAAGGACAGAAGTGAAGAAATCAAGAACTTGACTATCCCAGCCTT				800
Query 189	CTTCTCCGAATATGGATGTAATGCTAACCCTCCACGTTTGTTCGAAGAAATGGTACCTT				248
Sbjct 801	CTTCTCCGAATATGGATGTAATGCTAACCCTCCACGTTTGTTCGAAGAAATGGTACCTT				860
Query 249	GTATTCCAATAAAATGACTGATGTTTGGTCC 279				
Sbjct 861	GTATTCCGATAAGATGACTGATGTTTGGTCC 891				

Figura/Figure 4.10 Representación gráfica de la secuencia del clon N1 y comparación con la secuencia genómica de *C. albicans* que mostraba la mayor homología en las bases de datos consultadas.

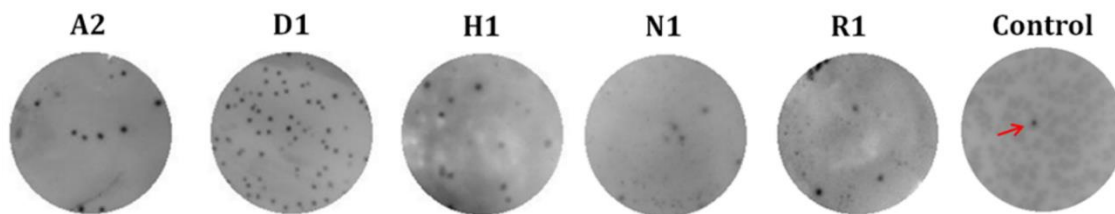
Candida albicans SC5314 chromosome 1 sequence					Clon R1
Sequence ID: CP017623.1 Length: 3188341 Number of Matches: 1					
Range 1: 2875464 to 2875993 GenBank Graphics			▼ Next Match ▲ Previous Match		
Score	Expect	Identities	Gaps	Strand	
974 bits(527)	0.0	529/530(99%)	0/530(0%)	Plus/Plus	
Query 169	CTTAAAAATAACCTTATCAAAATCTAAATTCCTATAAAAAACAATAAACATAACCTTAT				228
Sbjct 2875464	CTTAAAAATAACCTTATCAAAATCTAAATTCCTATAAAAAACAATAAACATAACCTTAT				2875523
Query 229	AACCTATCTATCATTTTGTAGATGTTTTCAAGAGCACTCTTTTTAATTGGTTCGTCATAC				288
Sbjct 2875524	AACCTATCTATCATTTTGTAGATGTTTTCAAGAGCACTCTTTTTAATTGGTTCGTCATAC				2875583
Query 289	TTGAGTATATCTTTACTCAAGTCATTAATTTTGCCAGTGGCCAACCAACAAAGTCACTT				348
Sbjct 2875584	TTGAGTATATCTTTACTCAAGTCATTAATTTTGCCAGTGGCCAACCAACAAAGTCACTT				2875643
Query 349	TGTAAACCTAAACTAATATCATCACCTTTACTCTCTTGCTAATTTCTTCAATTTAGAG				408
Sbjct 2875644	TGTAAACCTAAACTAATATCATCACCTTTACTCTCTTGCTAATTTCTTCAATTTAGAG				2875703
Query 409	GTTTGTTCATATGCCTTAGCTGCAATTTGCGCATTATCAACAATTGCATCAGGAATACCA				468
Sbjct 2875704	GTTTGTTCATATGCCTTAGCTGCAATTTGCGCATTATCAACAATTGCATCAGGAATACCA				2875763
Query 469	CACATGGATGCAACATTCATACCAACGACTTAGGTGCTGTTCTGTTTCCAACCTTGAT				528
Sbjct 2875764	CACATGGATGCAACATTCATACCAACGACTTAGGTGCTGTTCTGTTTCCAACCTTGAT				2875823
Query 529	AAAAATGTGATATTACGCGAGCCACTGTCAACAACGATACCCATTCTTAGTTGTTTGATC				588
Sbjct 2875824	AAAAATGTGATATTACGCGAGCCACTGTCAACAACGATACCCATTCTTAGTTGTTTGATC				2875883
Query 589	TGTGGGTGAGTCTTGAACGATAATCCCAAAGTACCATAAATGAGTAGCGAAAAATCCTAAT				648
Sbjct 2875884	TGTGGGTGAGTCTTGAACGATAATCCCAAAGTACCATAAATGAGTAGCGAAAAATCCTAAT				2875943
Query 649	GACTGAACATGGGTAGCTAAATGGTGTAAAAGTATTCGGCAATAGCAAA 698				
Sbjct 2875944	GACTGAACATGGGTAGCTAAATGGTGTAAAAGTATTCGGCAATAGCAAA 2875993				

Figura/Figure 4.11 Representación gráfica de la secuencia del clon R1 y comparación con la secuencia genómica de *C. albicans* que mostraba la mayor homología en las bases de datos consultadas.

Una vez comprobado que cada uno de los clones representaba un fragmento de ADN diferente de *C. albicans*, se confirmó la reacción de todos ellos con los CAGTA purificados



del conejo 167. Como se puede observar en la Figura/Figure 4.12, los CAGTA purificados reaccionaron con las proteínas expresadas por todos los clones seleccionados, destacando por su mayor intensidad los clones A2 y D1.



Figura/Figure 4.12 Clones positivos mediante inmunodetección con CAGTA purificados a partir de suero en un modelo de candidiasis invasora en conejo. Se muestra un control correspondiente a una suspensión de fagos al azar. La flecha roja indica la presencia de un clon positivo en la suspensión de fagos.

A partir de la secuencia de nucleótidos se derivó su secuencia de aminoácidos y se procedió a identificar la proteína codificada por cada uno de los clones con la herramienta informática Blast ofertada por el NCBI. En la Tabla/Table 4.1 se muestra la identidad más probable de las proteínas de *C. albicans* correspondientes a los clones seleccionados.

Tabla/Table 4.1 Identificación potencial de las proteínas de *C. albicans* codificadas por los 5 clones seleccionados por reaccionar con CAGTA.

Clon	Gen ^a	Nombre Proteína ^a	% Identidad ^b	Número de acceso (GenBank) ^c	Especie
A2	<i>PGI1</i>	Glucose-6-P-isomerase	100	XP_713554.1	<i>Candida albicans</i> SC5314
D1	<i>Hwp1</i>	Hyphal wall protein	100	XP_709961.1	<i>Candida albicans</i> SC5314
H1	<i>Fas1</i>	Fatty Acid Synthase	99	XP_716877.1	<i>Candida albicans</i> SC5314
N1	<i>Phr1</i>	pH-responsive protein 1	87	AAF73430.1	<i>Candida albicans</i> SC5314
R1	<i>Act1</i>	--	100	XP_722325.1	<i>Candida albicans</i> SC5314

^a Nombre del gen y de la proteína según la base de datos UniProtKB (Universal Protein Resource Knowledgebase).

^b % de cobertura de acuerdo al Blast.

^c Número de acceso en GenBank, NCBI (<http://www.ncbi.nlm.nih.gov/genbank/>).

A cada uno de los clones se les volvió a realizar una PCR. Los tamaños de los insertos varían hasta 4500 pb (Figura/Figure 4.13).

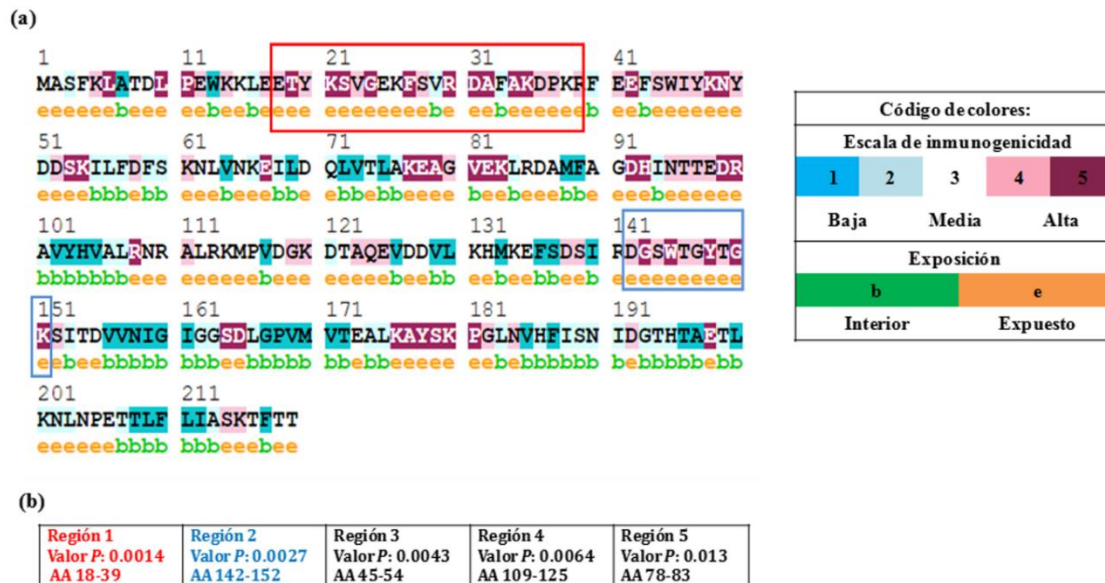


Figura/Figure 4.13 Amplificación por PCR de los clones positivos reaislados tras su reconocimiento por los CAGTA purificados. Las calles A2-R1 se corresponden a los clones positivos; la calle C- se corresponde al control negativo de la PCR.

4.2.2 Localización de regiones inmunogénicas

Las secuencias peptídicas codificadas por los clones seleccionados se analizaron con el programa Epitopia (Rubinstein, Mayrose, Martz, & Pupko, 2009) o, en el caso del clon D1, mediante el estudio inmunogénico con el algoritmo de Kolaskar y colaboradores (Kolaskar & Tongaonkar, 1990), con el fin de identificar las posibles regiones inmunogénicas teóricas reconocidas por los CAGTA.

El clon A2, que mostró un 100% de homología con la glucosa-6-fosfato isomerasa, codifica una secuencia de 219 aminoácidos en la que se identificaron cinco regiones antigénicas (Figura/Figure 4.14). Las regiones delimitadas por los aminoácidos 18-39 y 142-152 fueron las más significativas en la predicción de antigenicidad.

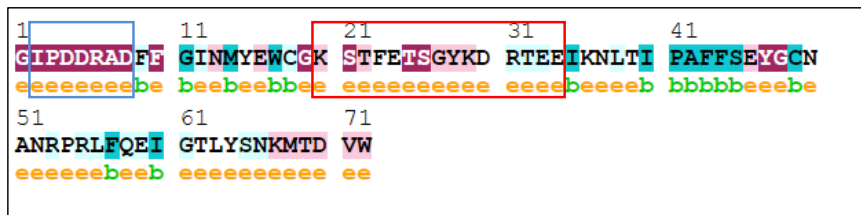


Figura/Figure 4.14 (a) Caracterización de regiones inmunogénicas del péptido codificado por el clon A2, mediante la herramienta informática Epitopia. En rojo y azul se resaltan las dos secuencias con mayor antigenicidad teórica. (b) Regiones con mayor grado de inmunogenicidad del péptido codificado por el clon A2 y reconocido por los CAGTA.

El péptido correspondiente al clon H1 reconocido por los CAGTA presenta un 100% de homología con el enzima ácido graso sintasa (Fas1). El estudio inmunogénico de los 236 aminoácidos reveló la presencia de 5 zonas inmunogénicas como se puede observar en la Figura/Figure 4.15.



(a)



(b)

Región 1 Valor P: 0.0156 AA 20-34	Región 2 Valor P: 0.03287 AA 02-08	Región 3 Valor P: 0.0338 AA 63-73	Región 4 Valor P: 0,1345 AA 47-48	Región 5 Valor P: 0.1762 AA 51-56
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Figura/Figure 4.16 (a) Caracterización de regiones inmunogénicas del péptido codificado por el clon N1 mediante la herramienta informática Epitopia. En rojo y en azul se resaltan las dos secuencias con mayor antigenicidad (b) Regiones con mayor grado de inmunogenicidad del péptido codificado por el clon N1 y reconocido por los CAGTA. Se ha aplicado el mismo código de colores de la Figura/Figure 4.14.

En el caso del clon R1, codifica una secuencia peptídica de 129 aminoácidos correspondientes a la proteína Act1 de *C. albicans*. El estudio de antigenicidad, como se puede observar en la Figura/Figure 4.17, refleja la presencia de 5 zonas inmunogénicas.

(a)



(b)

Region 1 Valor P: 0.0018 AA 2-13	Region 2 Valor P: 0.0065 AA 31-39	Region 3 Valor P: 0.0177 AA 18-23	Region 4 Valor P: 0.0214 AA 90-100	Region 5 Valor P: 0.0418 AA 108-112
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Figura/Figure 4.17 (a) Caracterización de regiones inmunogénicas del péptido codificado por el clon R1 mediante la herramienta informática Epitopia. En rojo y en azul se resaltan las dos secuencias con mayor antigenicidad (b) Regiones con mayor grado de inmunogenicidad del péptido codificado por el clon R1 y reconocido por los CAGTA. Se ha aplicado el mismo código de colores de la Figura/Figure 4.14.



La proteína codificada por el clon D1 presenta un 100% de homología con un fragmento de 309 aminoácidos de la proteína Hwp1 de *C. albicans*. Mediante el estudio inmunogénico con el algoritmo de Kolaskar y colaboradores, se ha observado la presencia de 12 posibles regiones antigénicas (Tabla/Table 4.2).

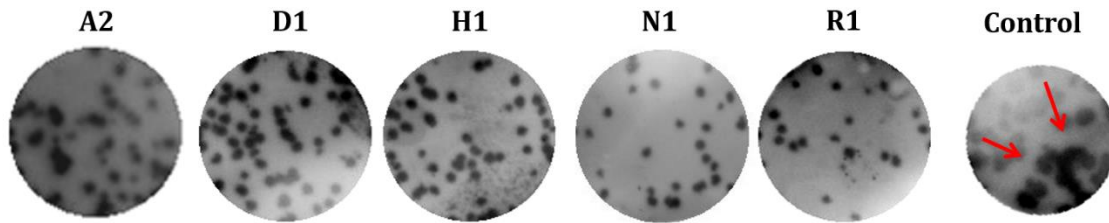
Tabla/Table 4.2 Predicción de regiones inmunogénicas del péptido codificado por el clon D1 mediante el algoritmo de Kolaskar y colaboradores.

Región	AA inicial	AA final	Secuencia	Longitud
1	8	14	QEPCDYP	7
2	22	28	PCDYPQQ	7
3	31	39	QEPCDYPQQ	9
4	41	49	QEPCDYPQQ	9
5	51	59	QEPCDYPQQ	9
6	69	77	QPDVPCDNP	9
7	79	87	QPDVPCDNP	9
8	90	97	PDIPCDNP	8
9	100	107	PDIPCDNP	8
10	183	188	SSVPAT	6
11	209	222	ESSVPVTSGSSILA	14
12	261	269	TTVVTVTSC	9

4.2.3 Reacción de los CAGTA de pacientes con candidiasis invasora

Con el fin de verificar una posible correspondencia entre el modelo animal de candidemia y la respuesta de pacientes con candidiasis invasora, se ensayó la reacción de la fracción enriquecida en CAGTA (CAGTA-enr) de un pool de sueros de cuatro pacientes con CI frente a los péptidos codificados por los 5 clones de la genoteca de *C. albicans* seleccionados a partir del modelo animal.

En la Figura/Figure 4.18 se puede observar una reacción muy intensa de la fracción CAGTA-enr de pacientes con CI frente a todos los clones aislados. En el grupo control se observó una reacción marcada, indicada con una flecha roja. En este, se incluyó una suspensión de fagos clones positivos a los CAGTA (en relación 1:10) intentando discriminar estos últimos de la suspensión de fagos al azar; siendo *Candida* un hongo colonizador habitual del hombre y siendo los CAGTA-enr menos específicos que los CAGTA purificados.



Figura/Figure 4.18 Reacción de los clones positivos seleccionados con la fracción CAGTA-enr obtenidos a partir de un pool de sueros de pacientes con CI. Se incluyó un control de fagos de la genoteca complementado con un 10% de los fagos seleccionados a partir del modelo animal de candidiasis.

4.3 Actividad biológica frente a *Candida albicans* de los anticuerpos desarrollados en un modelo de candidiasis invasora en conejo

Effect of antibodies developed in a rabbit model of candidemia on *Candida albicans* planktonic cells

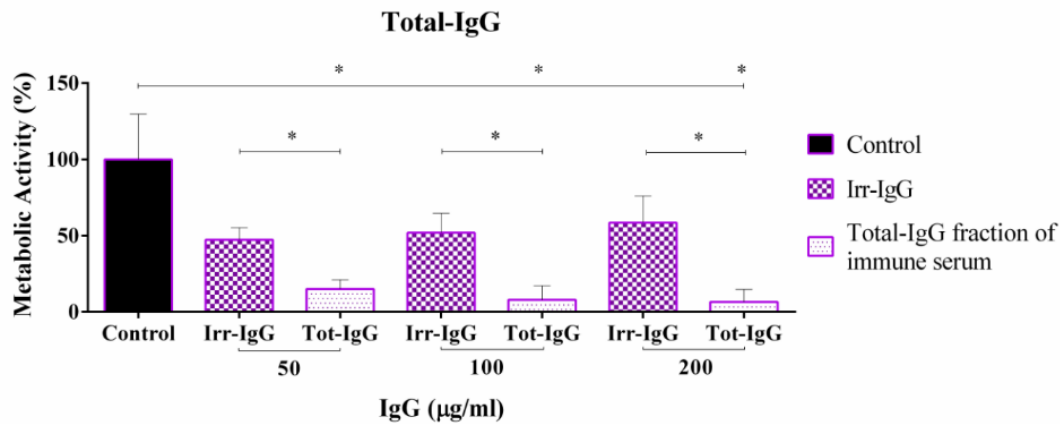
Los CAGTA, además de colaborar en el diagnóstico de la CI, según algunos estudios pueden ser de utilidad como marcadores pronósticos en pacientes con CI ingresados en la unidad de cuidados intensivos (UCI), observándose una reducción de la tasa de mortalidad en aquellos pacientes con títulos crecientes de CAGTA, siendo ésta del 22,7% en pacientes CAGTA positivos frente a 61,2 % en pacientes CAGTA negativos (Zaragoza et al., 2009b). Con el fin de caracterizar la actividad de los anticuerpos desarrollados por estos pacientes, se estudió inicialmente la capacidad de las distintas fracciones de anticuerpos desarrollados en un modelo de candidiasis en conejo, para inhibir el crecimiento y la actividad metabólica de *C. albicans*. De forma paralela, se realizó un estudio complementario con los sueros de un pequeño grupo de pacientes con candidiasis invasora. Este trabajo se ha enviado para su publicación a la revista Iberoamericana de Micología.

4.3.1 Effect on metabolic activity

Candida albicans yeast cells incubated with total-IgG from rabbit immune sera showed a drastic reduction of their metabolic activity, as evaluated by the XTT assay; the effect was concentration dependent (Figura/Figure 4.19). The effect resulted in a significant reduction down to 25% compared to untreated cells (control) and to cells treated with an equivalent amount of a commercialized irrelevant rabbit IgG (Irr-IgG). The metabolic



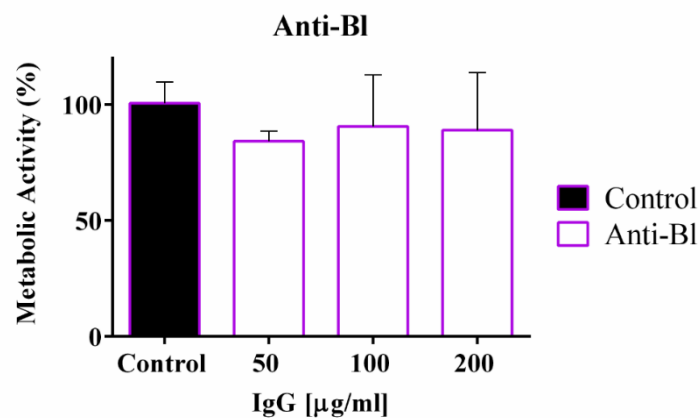
activity in presence of an irrelevant IgG was reduced, although it had not a concentration dependent effect.



Figura/Figure 4.19 Metabolic activity of *C. albicans* SC5314 yeast cells treated for 2.5 h with total serum IgG from a rabbit with invasive candidiasis. Metabolic activity measured with XTT assay. Bars represent mean value \pm SD of three independent experiments. Statistical significance $*P < 0.05$.

In order to know which fraction of serum is responsible for the anti-fungal effect, two different experiments were run using IgG antibodies anti-blastospore (anti-BL) and those from the CAGTA enriched fraction of the serum (CAGTA-enr).

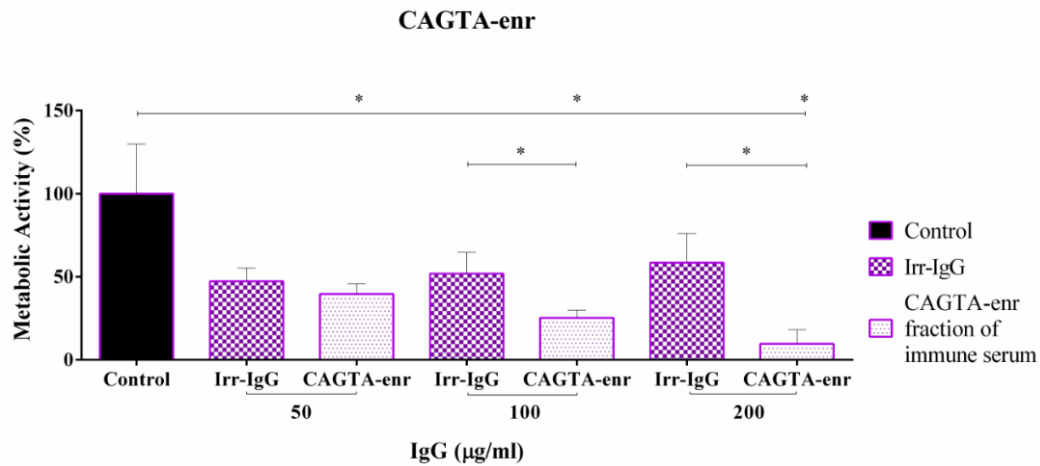
Anti-blastospores antibodies (anti-BL) ≤ 200 $\mu\text{g/ml}$ did not show any significant effect on the metabolic activity of the yeast cells (Figura/Figure 4.20).



Figura/Figure 4.20 Metabolic activity of *C. albicans* SC5314 yeast cells treated for 2.5 h with anti-Blastospore (Anti-BL) fraction of rabbit immune serum. Metabolic activity measured with XTT assay. Bars represent mean value \pm SD of three independent experiments.



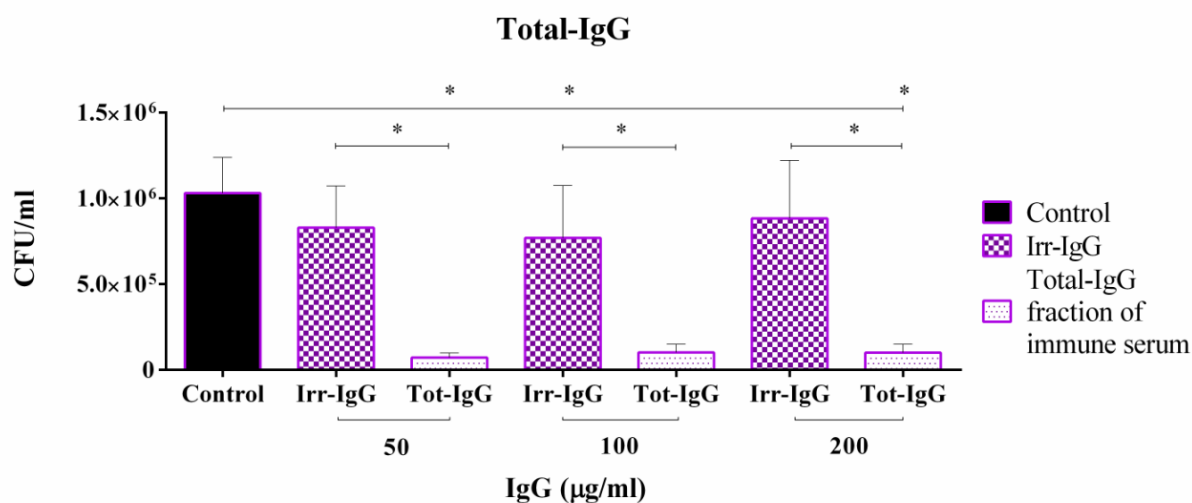
The experiment with IgG from the CAGTA enriched serum fraction (CAGTA-enr) gave a similar result to that of total-IgG (Figura/Figure 4.21) resulting in a reduction of *C. albicans* metabolic activity related to the IgG concentration.



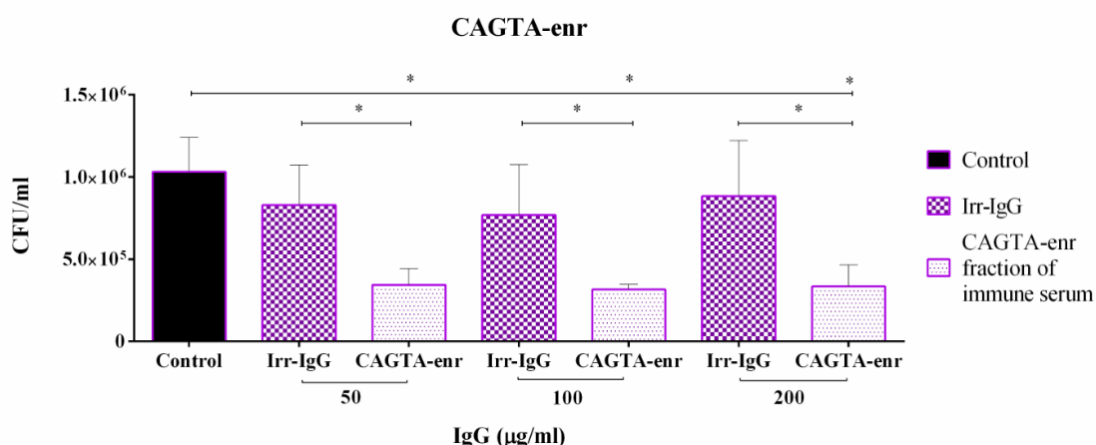
Figura/Figure 4.21 Metabolic activity of *C. albicans* SC5314 yeast cells treated for 2.5 h with CAGTA-enr fraction of rabbit immune serum. Metabolic activity measured with XTT assay. Bars represent mean value \pm SD of three independent experiments. Statistical significance $*P < 0.05$.

4.3.2 Colony forming unit assay (Effect on viability)

The effect of different serum fraction antibodies on metabolic activity of *C. albicans* planktonic cells was confirmed by the CFU assay, where Total-IgG and CAGTA-enr fractions caused a dramatic decrease in the number of colonies at IgG concentrations ≥ 50 $\mu\text{g/ml}$ (Figura/Figure 4.22 and Figura/Figure 4.23). The CFU experiment revealed that the cell viability was not affected by the Irr-IgG when compared to the untreated cells.



Figura/Figure 4.22 Viability *C. albicans* SC5314 treated for 2.5 h with total-IgG from immune serum of a rabbit with invasive candidiasis. Viability expressed as CFU. Bars represent mean value \pm SD of three independent experiments. Statistical significance $*P < 0.05$.



Figura/Figure 4.23 Viability *C. albicans* SC5314 treated for 2.5 h CAGTA-enr fraction from immune serum of a rabbit with invasive candidiasis. Viability expressed as CFU. Bars represent mean value \pm SD of three independent experiments. Statistical significance $*P < 0.05$.

4.3.3 Effect on growth and morphology

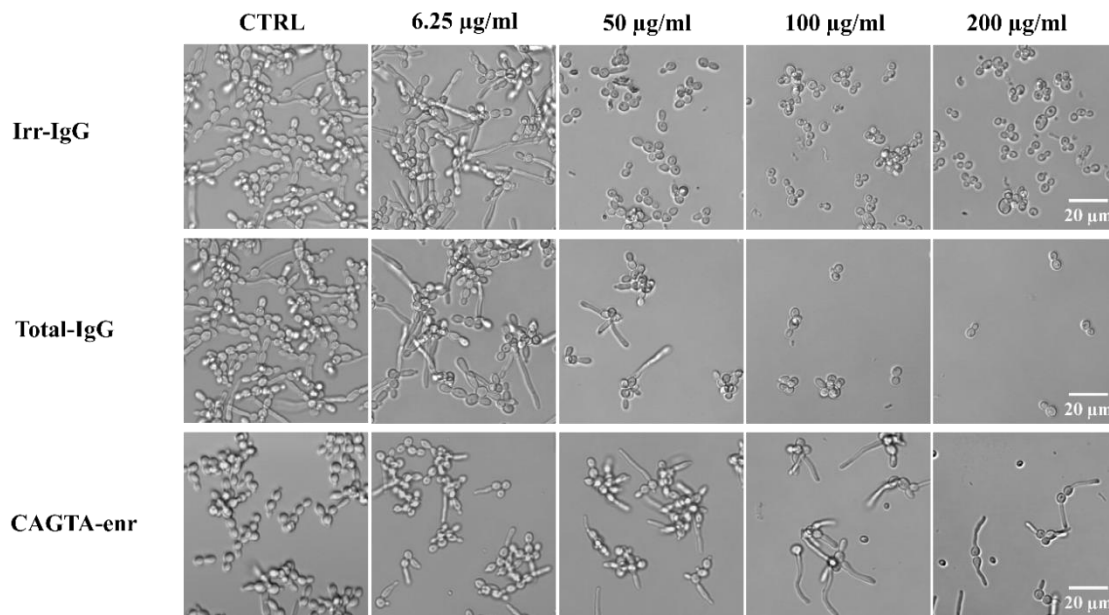
The optical microscopy images of *C. albicans* planktonic cells incubated with increasing concentrations of different IgG fractions of rabbit immune sera corroborated the above-mentioned findings (Figura/Figure 4.24).

Irr-IgG slightly reduced the growth of *Candida*. Total-IgG from rabbit immune serum reduced the growth of cells in a concentration dependent manner. CAGTA-enr for his part reduced *C. albicans* growth to a lesser extent and, even at the highest IgG concentration





200 $\mu\text{g/ml}$, short germ tubes were observed. This paradoxical effect did not correlate the results of XTT and CFU assays, but electron microscope images of *C. albicans* hyphae treated with CAGTA-enr IgG 40 $\mu\text{g/ml}$ revealed an altered surface (Figura/Figure 4.25: G-J). On the contrary, the surface of *C. albicans* untreated hyphae and yeast cells (Figura/Figure 4.25: A, B, E, and F) as well as that of blastospores treated with CAGTA (Figura/Figure 4.25: C, D) appeared regular and smooth.



Figura/Figure 4.24 Phase contrast microscopy images of *C. albicans* SC5314 grown at 37°C for 2.5 h in Sabouraud broth supplemented with different concentrations of irrelevant IgG (Irr-IgG), Total-IgG or CAGTA enriched serum fraction (CAGTA-enr). Control cells (CTRL) were incubated without antibodies.

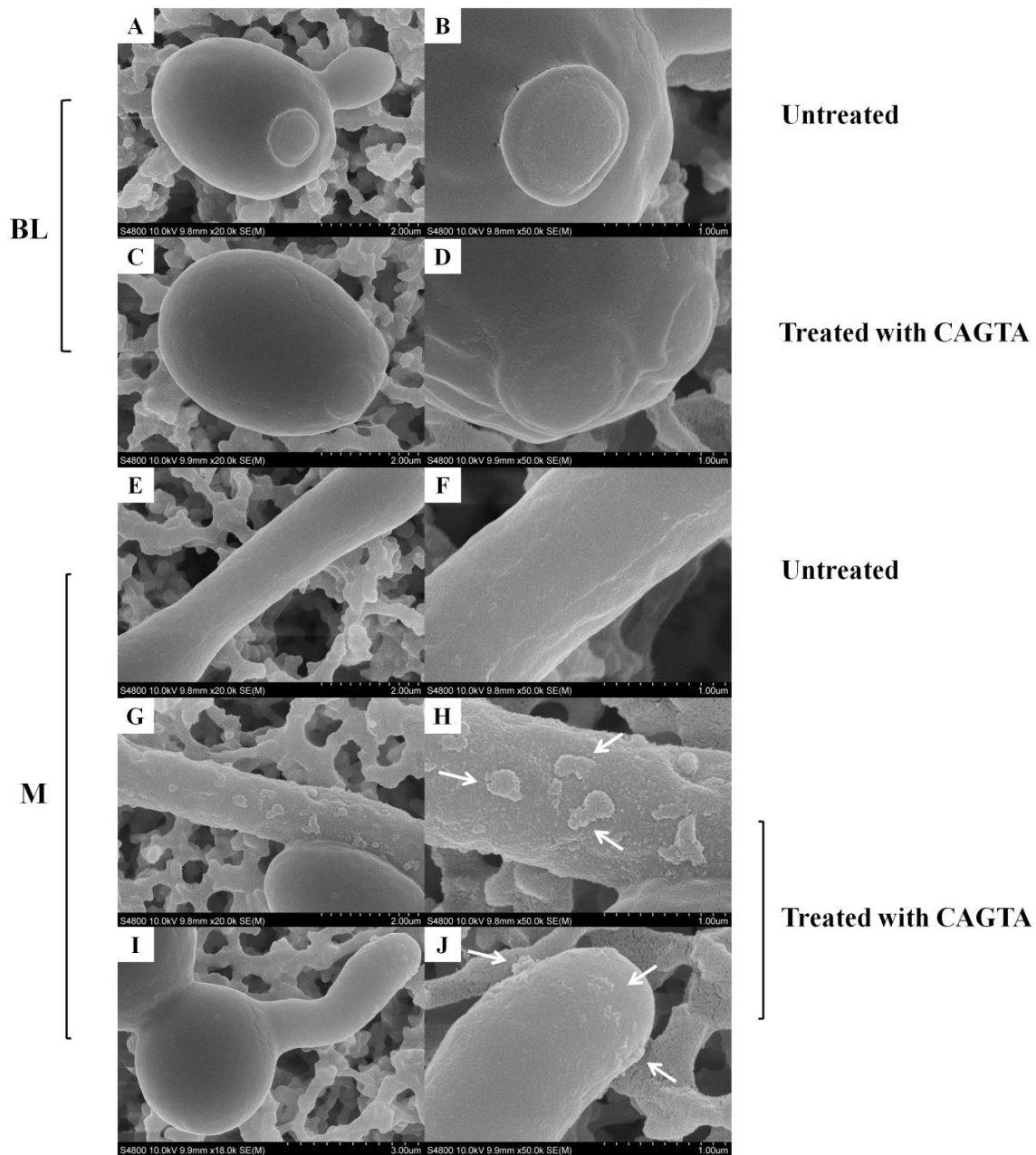
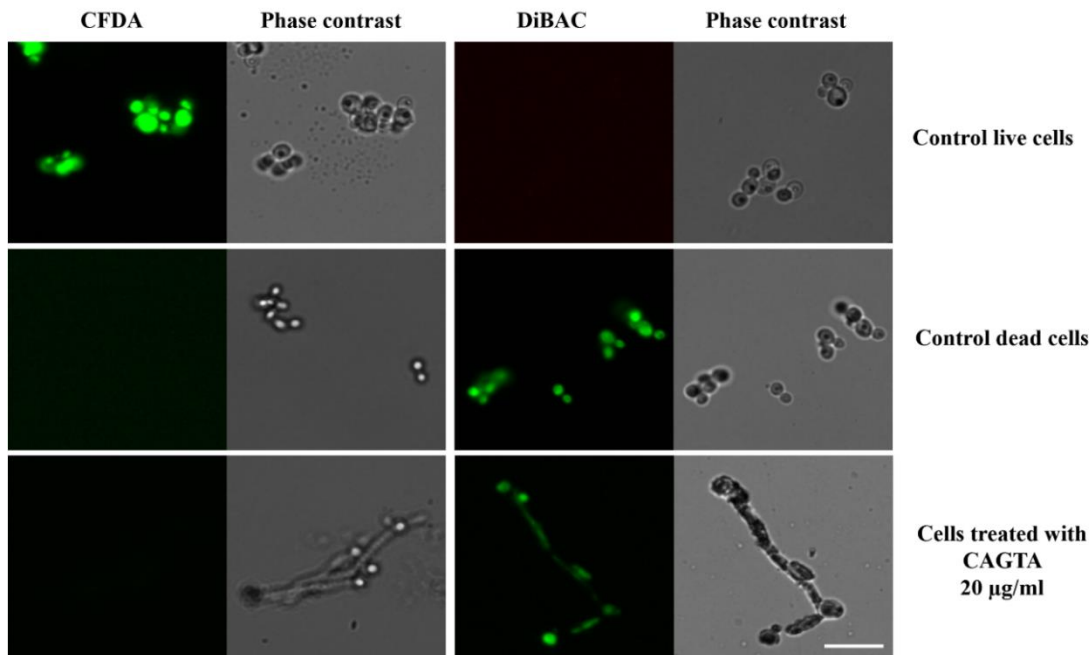


Figure 4.25 SEM images of *C. albicans* SC5314 blastospores (BL) and mycelia (M) grown at 37°C for 2.5 h in Sabouraud broth with CAGTA 40 µg/ml (C, D, G-J) or without antibodies (A, B, E and F). Magnification: x 20,000 (left column) and x 50,000 (right column) for details. Arrows highlight surface protuberances.

In addition, fluorescent DiBAC and CFDA staining of *C. albicans* cells treated with CAGTA 20 µg/ml revealed that these antibodies exerted a fungicidal effect (Figure 4.26).



Figura/Figure 4.26 Photomicrographs of *C. albicans* SC5314 germ tubes grown at 37°C for 2.5 h in Sabouraud broth supplemented with CAGTA 20 µg/ml and then stained with the fluorescent dyes CFDA and DiBAC. Staining control consisted of live and dead yeast cells that were grown without antibodies and, before staining, dead yeast cells were heat-killed at 60°C for 2 h. Paired images depict epifluorescence and phase contrast microscopy of the same field. Bar 20 µm.

4.4 Actividad biológica frente a *Candida albicans* de los anticuerpos desarrollados por pacientes con candidiasis invasora

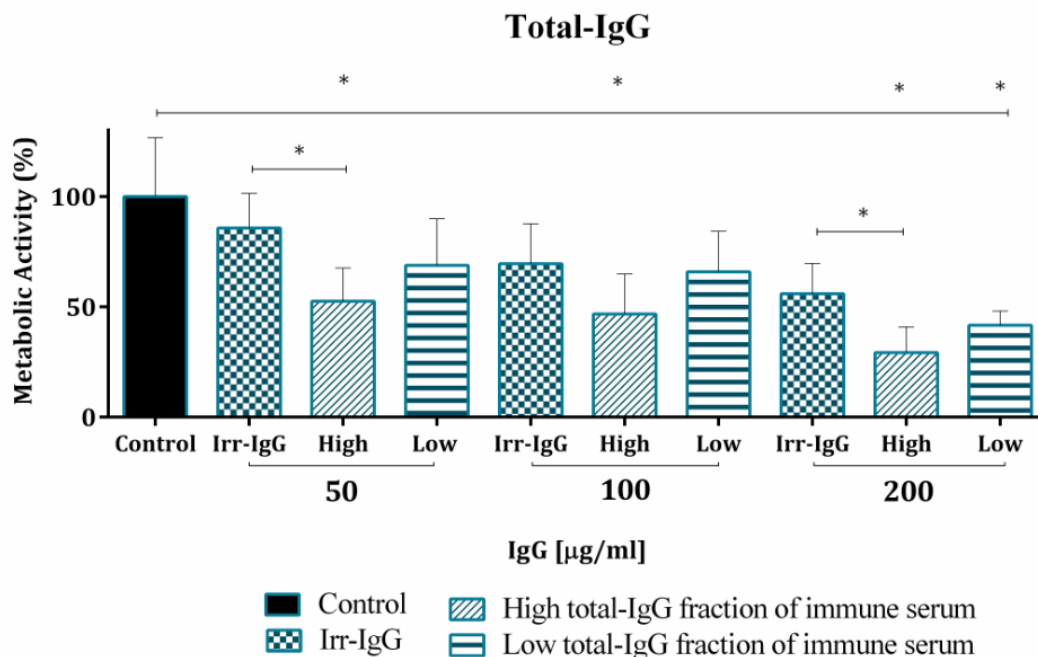
Effect of antibodies developed by patients with invasive candidiasis on *Candida albicans* planktonic cells

Based on the results obtained using antibodies from a rabbit infected by *C. albicans*; the same study was carried out using antibodies from patients infected by *C. albicans*.

4.4.1 Effect on metabolic activity of *Candida albicans* planktonic cells

Candida albicans yeast cells incubated with total-IgG from immune sera of patients with invasive candidiasis showed a marked reduction of their metabolic activity, as evaluated by the XTT assay, and the effect was concentration dependent (Figura/Figure 4.27).

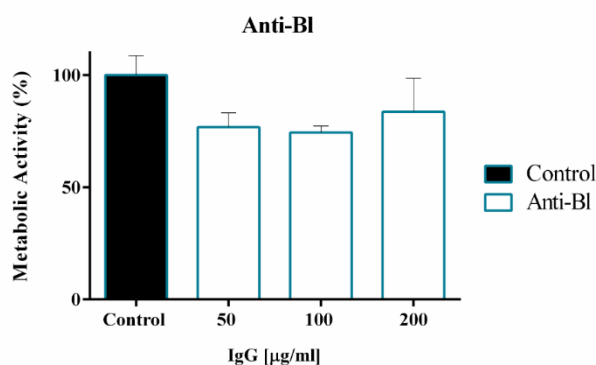
The effect of serum IgG from patients with high CAGTA titers resulted in a significant reduction of the metabolic activity down to 25-50% with respect to untreated cells. The effect of IgG from patients with low CAGTA titers was less potent but still higher than that of an equivalent amount of a commercialized irrelevant rabbit IgG (Irr-IgG). The metabolic activity in presence of an irrelevant IgG was slightly reduced.



Figura/Figure 4.27 Metabolic activity of *C. albicans* SC5314 yeasts treated for 2.5 h with total-IgG from sera (high and low CAGTA titer) of patients (groups I and II) with invasive candidiasis. Metabolic activity measured with XTT assay. Bars represent mean value ± SD from three independent experiments. Statistical significance * $P < 0.05$.

In order to know which fraction of serum is responsible for the anti-fungal effect, two different experiments were run using IgG antibodies anti-blastospore (anti-BI) and those from the CAGTA enriched fraction of the serum (CAGTA-enr).

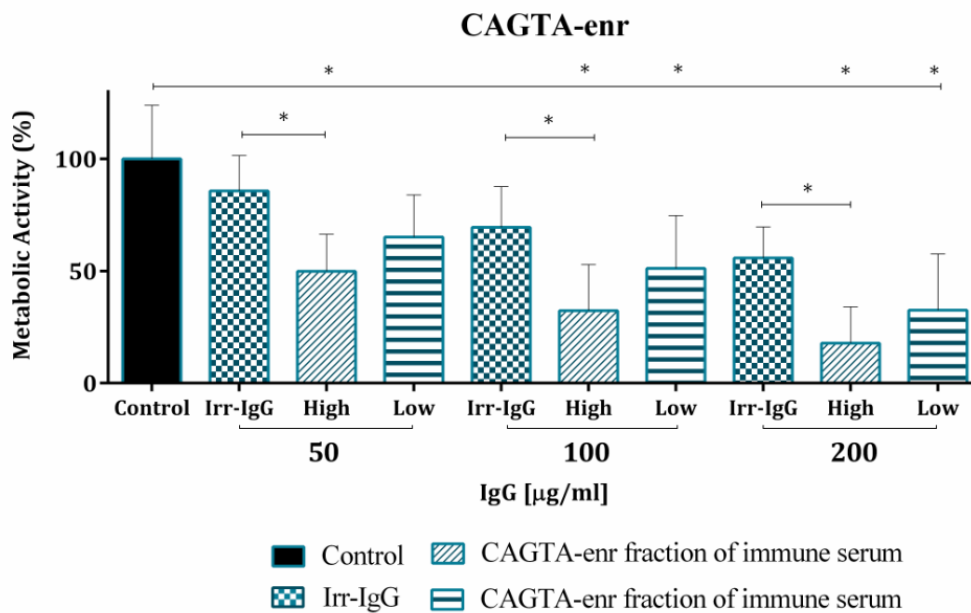
Anti-blastospores antibodies (anti-BL) ≤200 µg/ml did not show any significant effect on the metabolic activity of the yeast cells (Figura/Figure 4.28).



Figura/Figure 4.28 Metabolic activity of *C. albicans* SC5314 yeasts treated for 2.5 h with anti-Blastospore (Anti-BI) from sera (high and low CAGTA titer) of patients with invasive candidiasis (group I and II). Metabolic activity measured with XTT assay. Bars represent mean value ± SD from three independent experiments.



The experiment with IgG from the CAGTA enriched serum fraction (CAGTA-enr) gave a similar result to that of total-IgG (Figura/Figure 4.29) resulting in a reduction of *C. albicans* metabolic activity related to the IgG concentration.



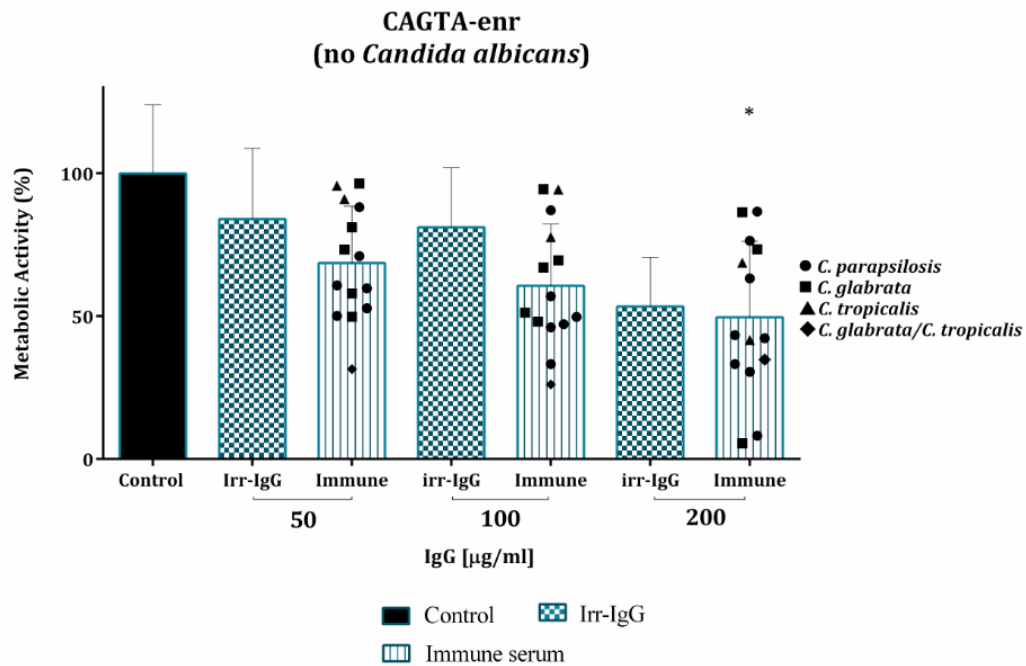
Figura/Figure 4.29 Metabolic activity of *C. albicans* SC5314 yeasts treated for 2.5 h with CAGTA-enr fraction from sera (high and low CAGTA titer) of patients with invasive candidiasis (group I and II). Metabolic activity measured with XTT assay. Bars represent mean value \pm SD from three independent experiments. Statistical significance $*P < 0.05$.

4.4.2 Effect of CAGTA-enr serum fraction of patients infected with *Candida non-albicans* species on the metabolic activity of *Candida albicans*

The effect of CAGTA-enr fraction of patients infected by *Candida albicans* was studied in the same way as the paragraph 4.4.1.

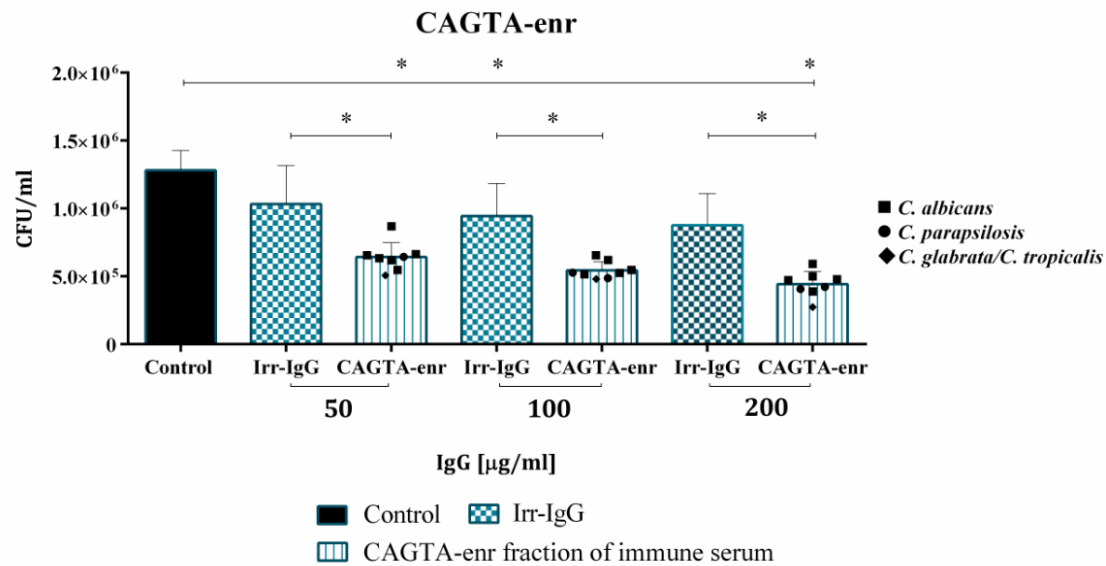
Serum CAGTA titers of 14 patients infected with *C. parapsilosis*, *C. glabrata* or *C. tropicalis* were $\leq 1/320$, excluding patient number 2 (group III) whose titer was $1/2560$.

As we can see in Figura/Figure 4.30, CAGTA-enr serum fractions of these patients reduced *C. albicans* metabolic activity to a different extent, with a mean value of 50-60% inhibition compared to the untreated control. Serum from patients infected with *C. tropicalis* showed the lowest effect.



Figura/Figure 4.30 Metabolic activity of planktonic cells of *C. albicans* SC5314 treated with CAGTA-enr serum fraction of patients (group III and IV) infected with *C. parapsilosis*, *C. glabrata*, *C. tropicalis* or *C. glabrata/C. tropicalis* for 2.5 h. Metabolic activity measured with XTT assay. Bars represent mean value \pm SD from three independent experiments. Statistical significance * $P < 0.05$.

The sera of patients showing the highest effect on metabolic activity also reduced the number of colony forming units of treated cells in comparison with the control groups (Figura/Figure 4.31). The CFU experiment revealed that cell viability was not affected by the Irr-IgG when compared to the untreated cells.



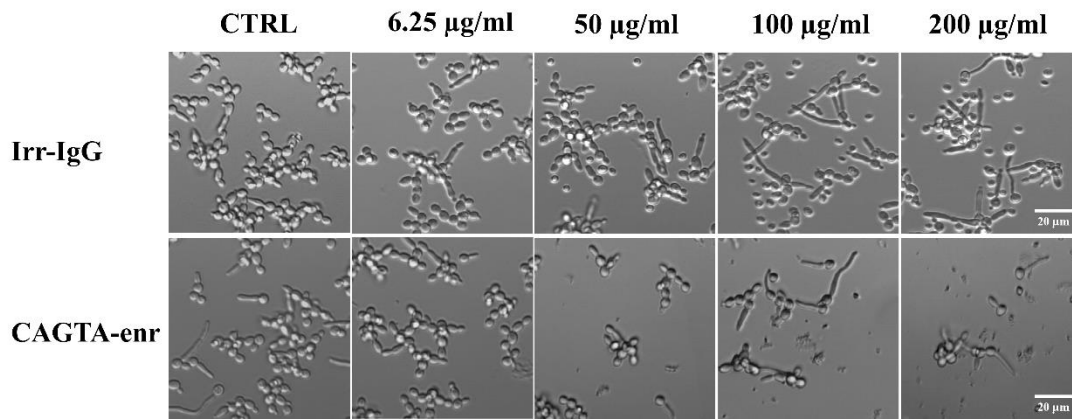
Figura/Figure 4.31 Effect of CAGTA-enr fraction from patient infected by *Candida* on planktonic cells. Viability of *C. albicans* SC5314 treated for 2.5 hours with CAGTA-enr serum fractions of patients (groups I-IV) infected with *Candida* species. Viability expressed as CFU. Bars represent mean value \pm SD from three independent experiments. Statistical significance $*P < 0.05$.

4.4.3 Effect on growth and morphology

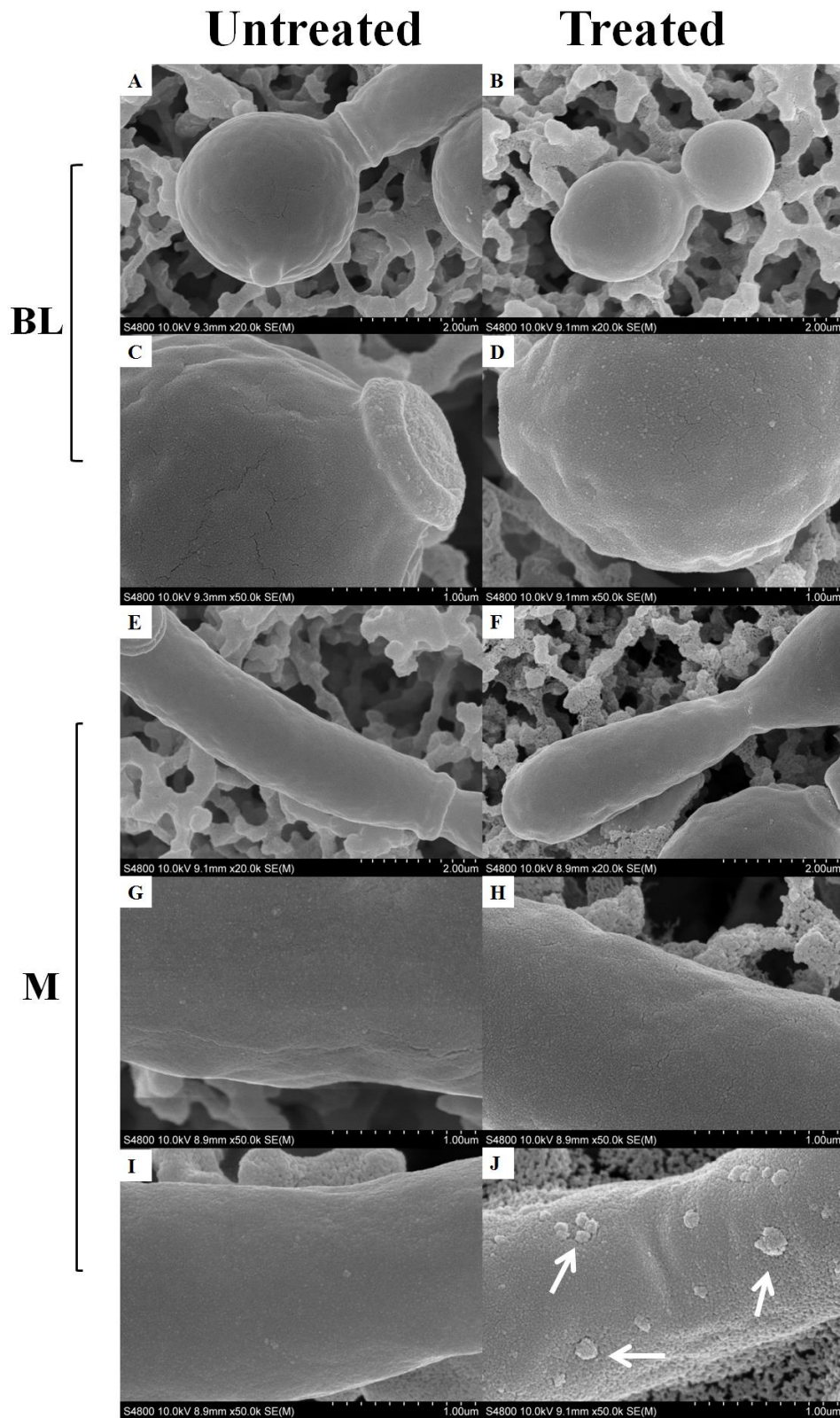
The optical microscopy images of *C. albicans* planktonic cells incubated with increasing concentrations of CAGTA-enr fractions of human immune sera corroborated the above-mentioned findings.

As we can observe in Figura/Figure 4.32, Irr-IgG slightly reduced the growth of *Candida*. For their part, CAGTA-enr IgG of patients' sera reduced *C. albicans* growth and, at the highest concentration 200 $\mu\text{g/ml}$, we still saw short germ tubes in the same way that we had observed with rabbit immune sera.

Electron microscope images of *C. albicans* treated with CAGTA-enr IgG 40 $\mu\text{g/ml}$ from patient #1 of group I (CAGTA titer 1/2560) revealed an altered surface of hyphae with fissures and protuberances (Figura/Figure 4.33: F, H, J). On the contrary, the surface of untreated control yeasts (Figura/Figure 4.33: A, C) and hyphae (Figura/Figure 4.33: E, G, I) as well as that of blastospores treated with CAGTA (Figura/Figure 4.33: B, D) appeared regular and smooth.



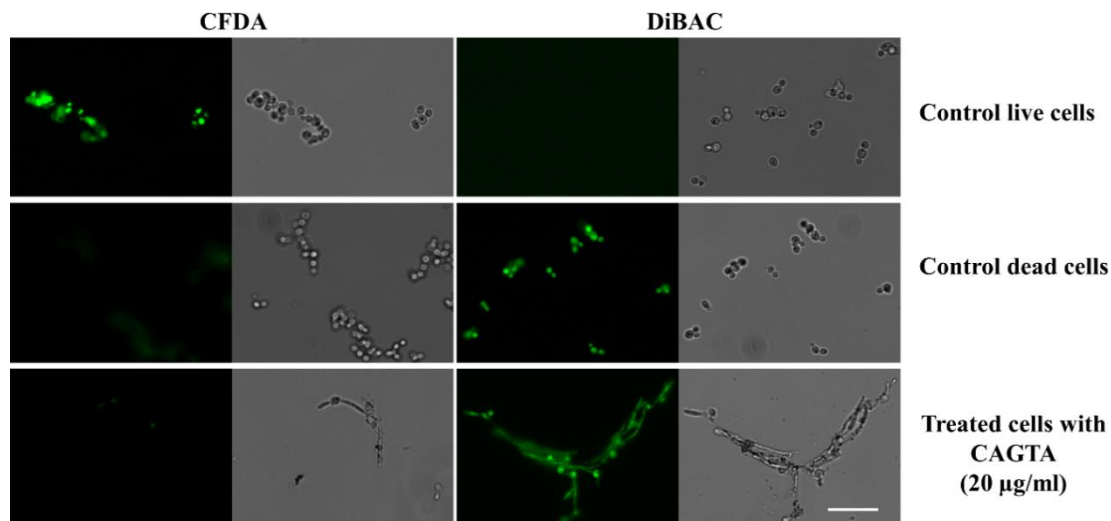
Figura/Figure 4.32 Phase contrast microscopy images of *C. albicans* SC5314 grown at 37°C for 2.5 h in Sabouraud broth supplemented with different concentrations of irrelevant IgG (Irr-IgG) and CAGTA enriched serum fraction (CAGTA-enr) of a patient #1 of group I (CAGTA titer 1/2560) with invasive candidiasis. Control cells (CTRL) were incubated without antibodies.



Figura/Figure 4.33 SEM images of *C. albicans* SC5314 blastospores (BL) and mycelia (M) grown at 37°C for 2.5 h in Sabouraud broth with CAGTA 40 µg/ml (right column) or without antibodies (left column). Magnification: x 20,000 (A, B, E and F) and x 50,000 (C,D,G-J). Arrows highlight surface protuberances.



In addition, fluorescent DiBAC and CFDA staining of *C. albicans* cells treated with purified human (patient #1 of group I) CAGTA 20 µg/ml revealed that these antibodies exerted a fungicidal effect (Figura/Figure 4.34).

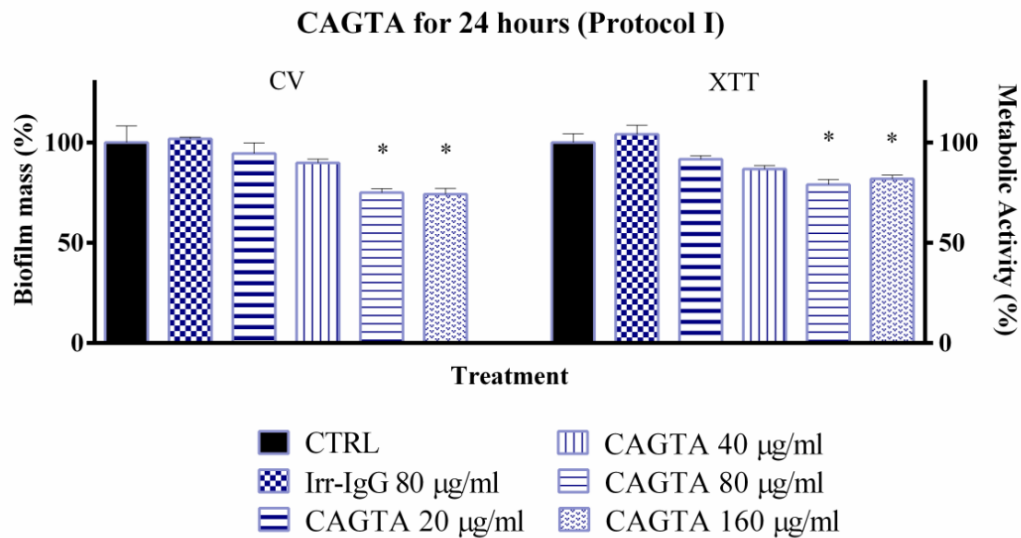


Figura/Figure 4.34 Photomicrographs of *C. albicans* SC5314 germ tubes grown at 37°C for 2.5 h in Sabouraud broth supplemented with CAGTA 20 µg/ml and then stained with the fluorescent dyes CFDA and DiBAC. Staining control consisted of live and dead yeast cells that were grown without antibodies and, before staining, dead yeast cells were heat-killed at 60°C for 2 h. Paired images depict epifluorescence and phase contrast microscopy of the same field. Bar 20 µm.

4.5 Effect of CAGTA raised in a rabbit model of invasive candidiasis on *Candida albicans* biofilm

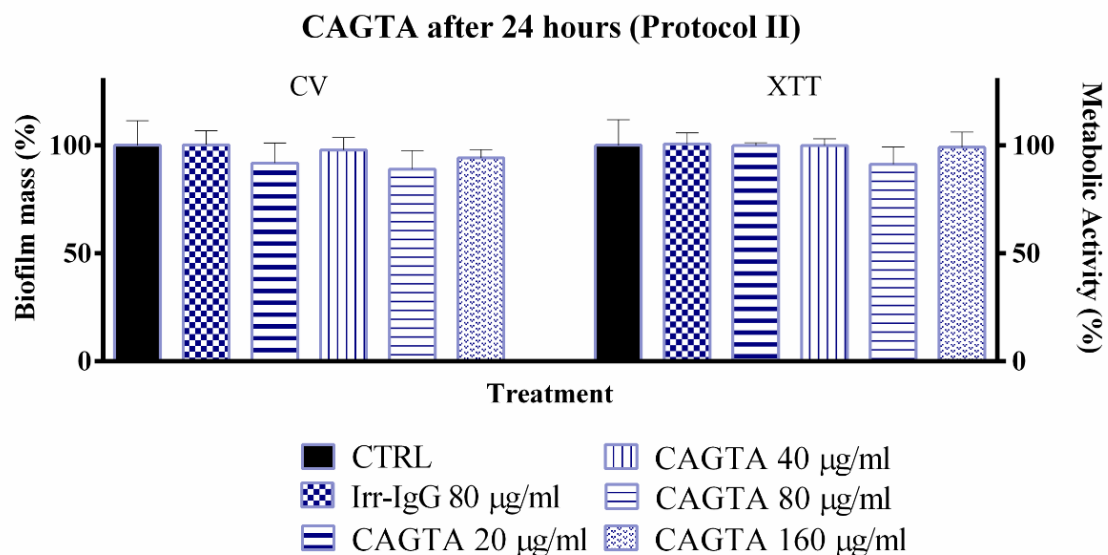
The ability of *C. albicans* to form biofilm is an important virulence factor. Since CAGTA showed an inhibitory effect on *C. albicans* planktonic cells, we decided to study the effect of CAGTA during the biofilm formation process.

The formation of biofilm by *C. albicans* can be divided in phases that were analyzed following different time-based protocols of treatment with CAGTA purified from serum of a rabbit with invasive candidiasis. *C. albicans* yeast cells exposed to CAGTA (20-160 µg/ml) for the first 24 h of incubation (Protocol I), experienced a statistically significant reduction of biofilm production at CAGTA concentrations ≥ 80 µg/ml (Figura/Figure 4.35), while no differences were observed at the lowest CAGTA concentrations or in the presence of the irrelevant IgG (80 µg/ml). Similar profiles were obtained with the crystal violet (CV) estimation of biofilm mass and metabolic activity according to the XTT assay.



Figura/Figure 4.35 Effect of CAGTA on metabolic activity (XTT assay) and biofilm mass (CV assay) of *C. albicans* SC5314. Experimental protocol I is described in Material and Methods section 3.12. Bars represent mean value \pm SD of three independent experiments. Statistical significance $*P < 0.05$.

When 24 h-old preformed biofilm samples were supplemented with CAGTA (20-160 $\mu\text{g/ml}$), according to Protocol II, the maturation of the biofilm was not affected after an additional 24-h incubation period (Figura/Figure 4.36).

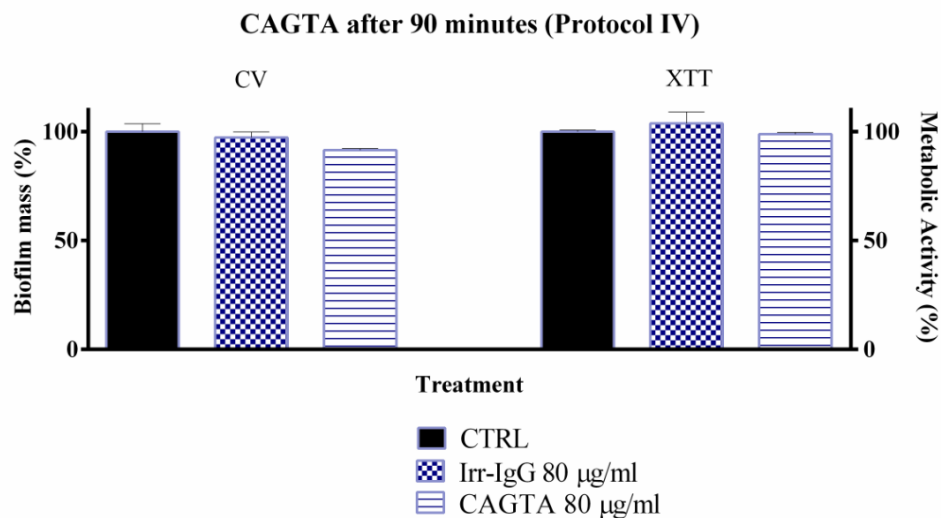


Figura/Figure 4.36 Effect of CAGTA on metabolic activity (XTT assay) and biofilm mass (CV assay) of *C. albicans* SC5314. Experimental protocol II is described in Material and Methods section 3.12. Bars represent mean value \pm SD of three independent experiments. Statistical significance $*P < 0.05$.

To further characterize the effect of CAGTA on *C. albicans*, two additional experimental protocols were performed focusing on the early phases of the biofilm formation process.

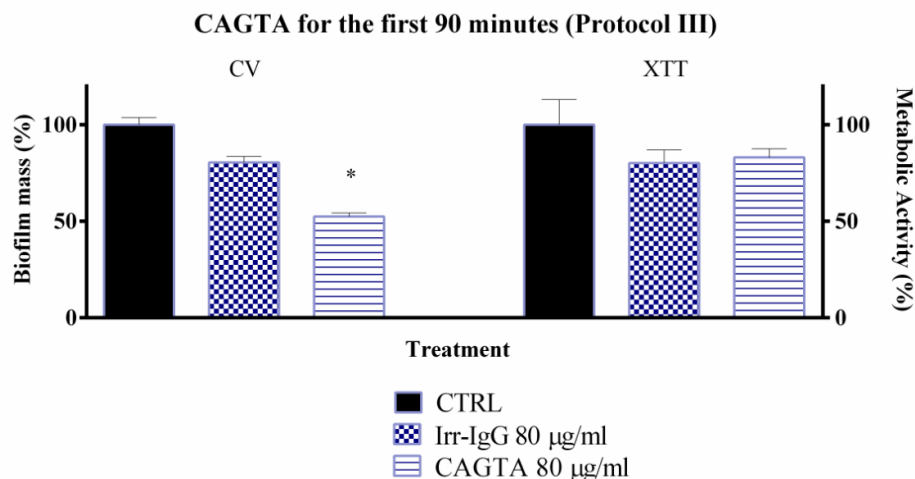


When CAGTA were added after the first 90 minutes of incubation, no significant effect on the development of the 24h-old biofilm was recorded (Protocol IV; Figura/Figure 4.37).



Figura/Figure 4.37 Effect of CAGTA on metabolic activity (XTT assay) and biofilm mass (CV assay) of *C. albicans* SC5314. Experimental protocol IV is described in Material and Methods section 3.12. Bars represent mean value \pm SD of three independent experiments. Statistical significance $*P < 0.05$.

However, the presence of CAGTA only during the first 90 minutes of incubation (Protocol III), corresponding to the *C. albicans* adhesion step, resulted in a significant reduction of the biofilm biomass -down to 48%- while the metabolic activity remained close to the control group values (Figura/Figure 4.38).

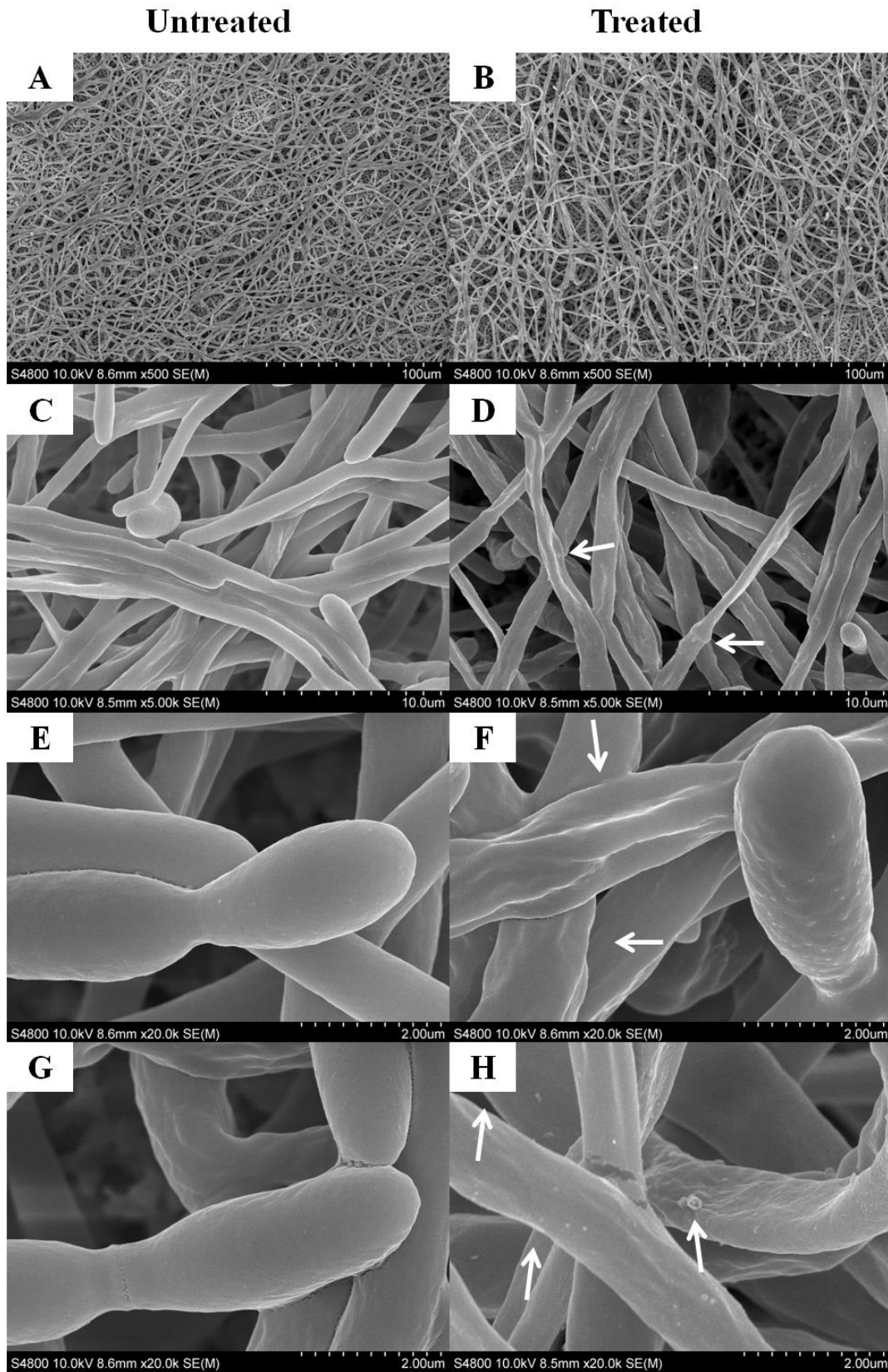


Figura/Figure 4.38 Effect of CAGTA on metabolic activity (XTT assay) and biofilm mass (CV assay) of *C. albicans* SC5314. Experimental protocol III is described in Material and Methods section 3.12. Bars represent mean value \pm SD of three independent experiments. Statistical significance $*P < 0.05$.



4.5.1 Morphological analysis

In accordance with the observed effect of CAGTA on *C. albicans* biofilm formation (Protocol I), electron micrographs of 24-hour old biofilms exhibited a reduction of the microbial structure density of treated cells (Figura/Figure 4.39: B), when compared to the untreated control (Figura/Figure 4.39: A). Moreover, micrographs revealed that CAGTA treatment had altered the cell wall surface that appeared rough and with protuberances (Figura/Figure 4.39: D, F and H), while the control group cells showed regular and smooth surfaces (Figura/Figure 4.39: C, E and G).



Figura/Figure 4.39 SEM images of 24-hour biofilm of *C. albicans* SC5314 developed at 37°C in RPMI+FBS10% with CAGTA 80 µg/ml (right panel) or without antibodies (left panel). Magnification: x 500 (A, B), x 5,000 (C, D) and x 20,000 (E-H). Arrows highlight the altered surface and protuberances of CAGTA treated cells.



4.6 Effect of Candidalysin of *Candida albicans* and *Candida albicans* mutant strains on TR146 epithelial cells line

The following experiments performed during a stage at Dr Naglik's laboratory (King's College, London; September-December 2016) aimed to build on these landmark discoveries to delineate the mechanistic function of the Candidalysin family.

Dr Naglik's recent discovery of Candidalysin, the first cytolytic peptide toxin identified in any human fungal pathogen, reveals a novel and unexpected factor by which *C. albicans* induces mucosal pathogenicity and immune activation (Moyes et al., 2016). Candidalysin (SIIGIIMGILGNIPQVIQIIMSIVKAFKGNK; Ece1-III_{62-92K}) is a peptide derived from the Ece1 protein of *C. albicans* SC5314. Ece1p is an *in vitro* substrate for Kex2p, a Golgi-located protease that cleaves proteins after lysine-arginine (KR) motifs. In fungi, it is known that following Kex2p processing, many proteins are subsequently cleaved by Kex1p29 (also in the Golgi), removing the C-terminal R. To confirm that removal of the C-terminal arginine residue from immature Candidalysin was due to the function of Kex1p, we analyzed the c-Fos binding activity of a *kex1Δ/Δ* null mutant compared to its matched revertant strain. c-Fos translocate to the nucleus and binds to its consensus DNA sequence site, which initiates the induction of downstream pro-inflammatory cytokines, including, IL-1 α , IL-6, G-CSF, GM-CSF and IL-1 β (Moyes et al., 2010).

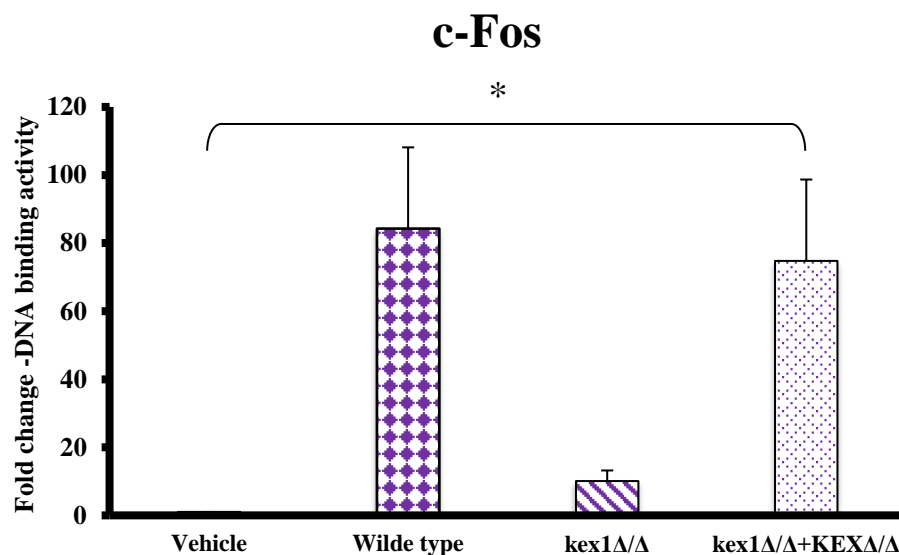
We know that the dominant peptide secreted from *C. albicans* hyphae during mucosal infection is Ece1-III_{62-92K}, which acts as a cytolytic peptide toxin that activates epithelial cells. Ece1-III_{62-92K} has α -helical structure and induces c-Fos, p-MKP1, damage (LDH), membrane intercalation and permeabilization, and calcium influx of TR146 line of epithelial cells, as well as the cytokines profile (IL-1 α , G-CSF) (Moyes et al., 2016). These authors postulated that the positively charged C-terminal K residue of Candidalysin might be critical for interacting with negatively charged components of host membranes and promoting damage. For this purpose, we obtained different Candidalysin molecules whose last amino acid K (lysine; positively charged) was substituted with H (histidine; positively charged), E (glutamic acid; negatively charged), or A (alanine; neutral charge) and their activity on TR146 epithelial cells was compared.



4.6.1 c-Fos activation by wild type and mutant strains of *Candida albicans*

We studied c-Fos activation of TR146 epithelial cells monolayers after being incubated with *C. albicans* wt (parental control) and mutant strains *kex1Δ/Δ* and *kex1Δ/Δ+KEX1* (re-integrant).

As we can observe in the Figura/Figure 4.40, the infection of TR146 epithelial cells with the wild type strain resulted in c-Fos DNA binding activation. While the infection of TR146 cells with *kex1Δ/Δ* did not result in c-Fos DNA binding activation, the exposure to the re-integrant strain *kex1Δ/Δ+KEX1* recovered the activation level induced by the parental strain. We observed statistically significant differences compared with the vehicle control.



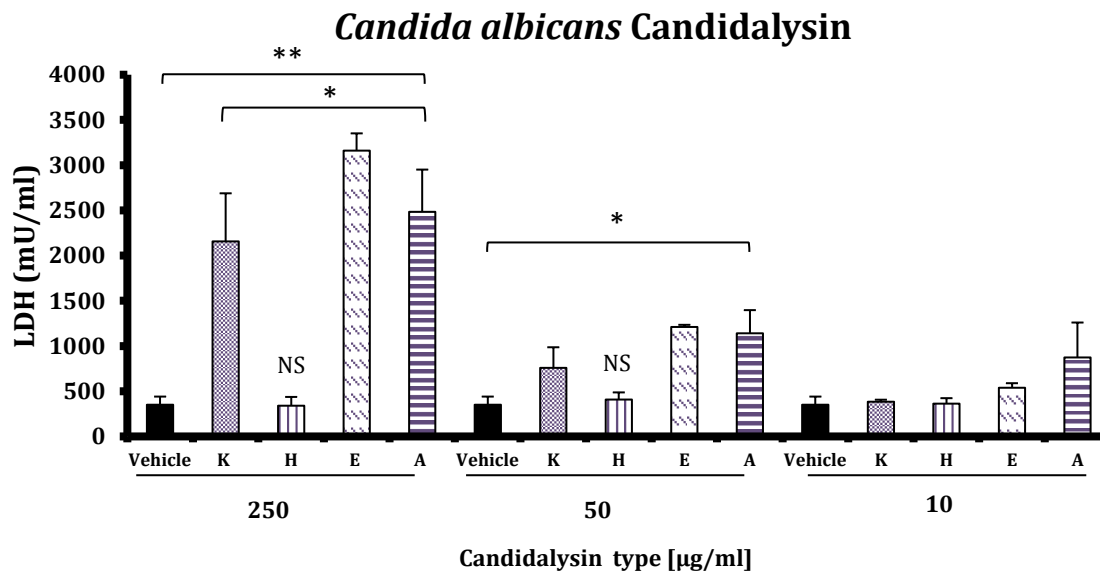
Figura/Figure 4.40 Analysis of c-Fos DNA binding activity in TR146 epithelial cells infected with *C. albicans* wt (parental control), *kex1Δ/Δ*, and *kex1Δ/Δ+KEXΔ/Δ* re-integrant. Data are represented as fold change relative to vehicle control. Bars represent the mean value of three biological replicates \pm SD. Statistical significance $*P < 0.05$.

4.6.2 LDH activity to estimate damage induced by *Candida albicans* Candidalysin variants

Candidalysin variants K, E and A induced a dose dependent cell damage estimated as LDH activity of TR146 epithelial cells, and the effect was statistically significant at concentrations ≥ 50 $\mu\text{g/ml}$, when compared with the no peptide control (vehicle) and ≥ 250 $\mu\text{g/ml}$ when compared with the peptide H. It is noticeable that peptide H-positively charged- showed no activity on the epithelial cells while the peptide E-negatively



charged- and the peptide A -neutral charge- unexpectedly produced more damage than the peptide K (Figura/Figure 4.41).

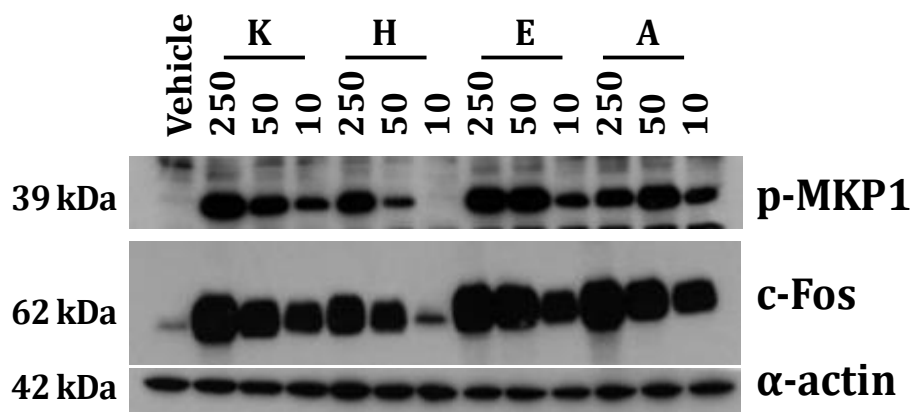


Figura/Figure 4.41 Quantification of lactate dehydrogenase (LDH) activity in the culture supernatant of TR146 epithelial cells to estimate the damage induced by *C. albicans* Candidalysin variants (K, H, E and A). The vehicle bar represents the control experimental conditions with no Candidalysin. Bars represent the mean value of three biological replicates \pm S.E.M. Statistical significance * $P < 0.05$, ** $P < 0.01$, NS not significant.

4.6.3 c-Fos and MKP-1 induction by Candidalysin variants of *Candida albicans*

We studied c-Fos and p-MKP1 proteins expression of TR146 epithelial cells monolayers after being incubated with *C. albicans* Candidalysin peptides K, H, E and A.

The four *C. albicans* Candidalysin variants induced a strong dose dependent expression of c-Fos and p-MKP1 compared with the vehicle control. Although the peptide H was able to induce the expression of both proteins, the effect was the weakest when compared with the other peptides. Unexpectedly the induction of p-MKP1 with peptide A at 50 $\mu\text{g/ml}$ was a little bit stronger than 250 $\mu\text{g/ml}$ (Figura/Figure 4.42).



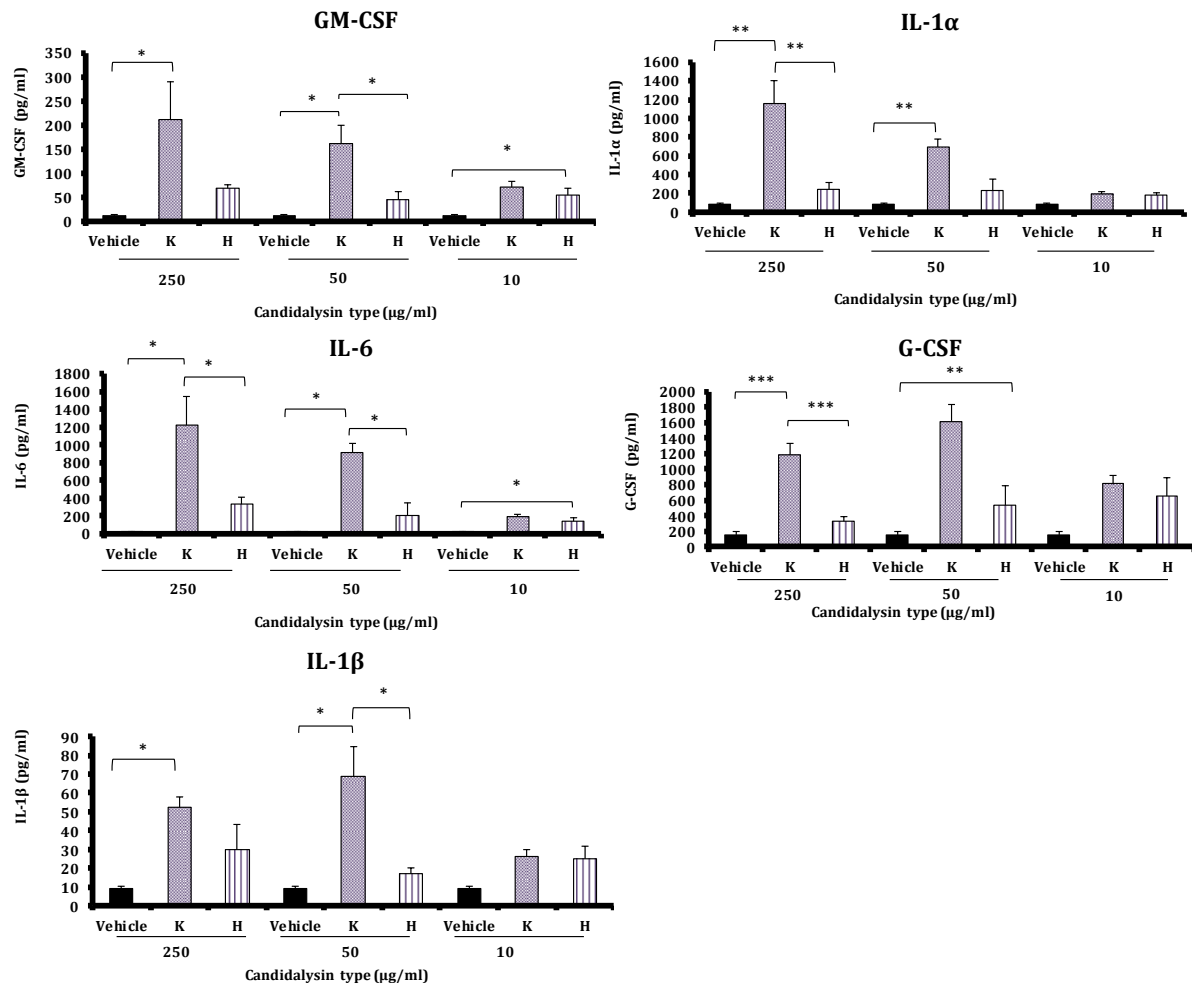
Figura/Figure 4.42 Induction of c-Fos and p-MKP1 after 2 h of incubation of TR146 epithelial cells with Candidalysin variants of *C. albicans* (K, H, E and A) at 250, 50 and 10 µg/ml. Vehicle lane represent the basal expression of untreated cells. Expression of α -actin is shown as a reference constitutive protein control. Data is representative of three biological replicates.

4.6.4 Production of cytokines in oral epithelial cells induced by *Candida albicans* Candidalysin variants.

C. albicans SC5314 Ece1p-III_K (Candidalysin) is a potent cytolytic agent essential for mucosal pathogenesis and immune activation *in vivo*. The activation of cytokines is mediated by c-Fos through p38 pathway, while the phosphorylation of MKP-1 is a regulator of the pro-inflammatory response mediated by ERK1/2 pathway (Moyes et al., 2010). In this experiment, we compared the production of several cytokines induced by the presence of the two Candidalysin variants with positively charged amino acids (K and H).

As we can see in Figura/Figure 4.43, both peptides induced the production of cytokines, although the levels reached in the presence of Candidalysin-K were more elevated than were those of the H-variant at concentrations ≥ 50 µg/ml.

IL-6, IL-1 α and GM-CSF were produced in a dose-dependent manner in the presence of increasing concentrations of Candidalysin. The increase was statistically significant for GM-CSF and IL-6 at concentrations of Candidalysins ≥ 10 µg/ml, and ≥ 50 µg/ml for IL-1 α . On the other side, the maximum response of G-CSF and IL-1 β production was reached in the presence of Candidalysin-K 50 µg/ml. Both peptides at low concentration (10 µg/ml) increased the production of these cytokines with reference to the control vehicle assay; however, the results for Candidalysin-K were only statistically significant at concentrations ≥ 50 µg/ml.



Figura/Figure 4.43 Secretion of GM-CSF, IL-1 α , IL-6, G-CSF and IL-1 β by TR146 epithelial cells line after incubation with Candidalysins K and H of *C. albicans* for 24h. Bars represent the mean value of three biological replicates \pm S.E.M. Statistical significance * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

4.7 Effect of Candidalysins of *Candida dubliniensis* and *Candida tropicalis* on TR146 epithelial cell line

C. tropicalis and *C. dubliniensis* are two emergent pathogens of the genus *Candida* that can induce epithelial damage (Moyes et al., 2012). *C. dubliniensis* and *C. albicans* are able to produce hyphae while *C. tropicalis* can at least produce pseudohyphae. According to JPred secondary structure software prediction (JPred 4.0), *C. albicans* and *C. dubliniensis* wild type Candidalysins present a double α -helix separated by a spacer region, while *C. tropicalis* Candidalysin shows a single α -helix without a spacer region. These Candidalysins are able to induce epithelial cells damage, but that of *C. tropicalis* up to now seems to be the most potent. In order to characterize the Candidalysins of *C. tropicalis*

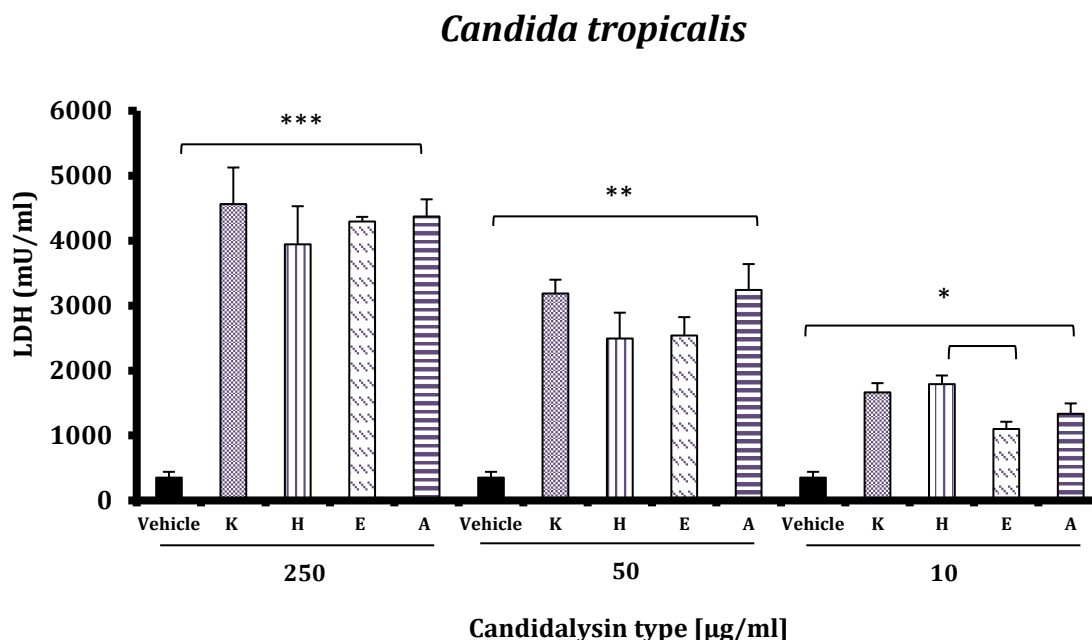


and *C. dubliniensis*, we ran experiments with K, H, E and A variants of their respective molecules, in a similar way to those performed with *C. albicans* Candidalysins.

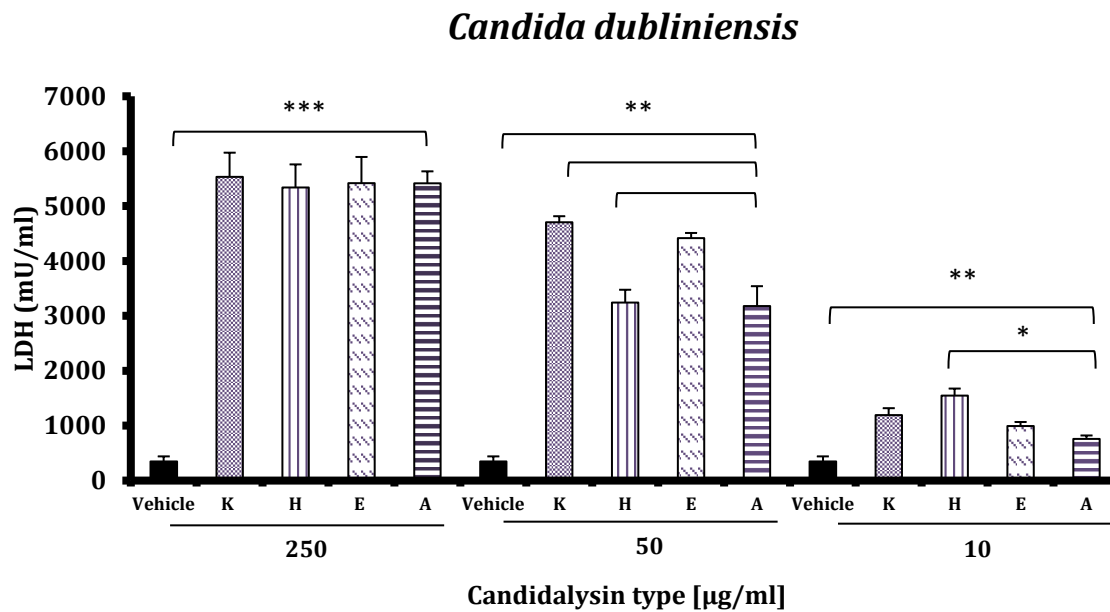
4.7.1 LDH activity to estimate damage induced by *Candida tropicalis* and *Candida dubliniensis* Candidalysins

C. tropicalis and *C. dubliniensis* Candidalysins induced a dose-dependent response in TR146 epithelial cells. The effect of *C. dubliniensis* Candidalysin was stronger than that of *C. tropicalis* for all the molecule variants assayed (Figura/Figure 4.44 and Figura/Figure 4.45). All Candidalysin variants at concentrations $\geq 10 \mu\text{g/ml}$ caused a statistically significant damage when compared with the control vehicle experimental condition. However, all molecules, even the A ending (neutral charge) peptide, rendered a similar effect to that of the wild variants (K). The *C. tropicalis* peptide H at $10 \mu\text{g/ml}$ caused a statistically significant damage when compared with the peptide E (Figura/Figure 4.44).

The *C. dubliniensis* peptide K at $50 \mu\text{g/ml}$ caused a statistically significant damage when compared with the peptide A, while the peptide H, at concentrations $\leq 50 \mu\text{g/ml}$ showed statistical difference in comparison with the peptide A (Figura/Figure 4.45).



Figura/Figure 4.44 Quantification of lactate dehydrogenase (LDH) activity in the culture supernatant of TR146 epithelial cells to estimate the damage induced by *C. tropicalis* Candidalysin variants (K, H, E and A). The vehicle bar represents the control experimental conditions with no Candidalysin. Bars represent the mean value of three biological replicates \pm S.E.M. Statistical significance * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

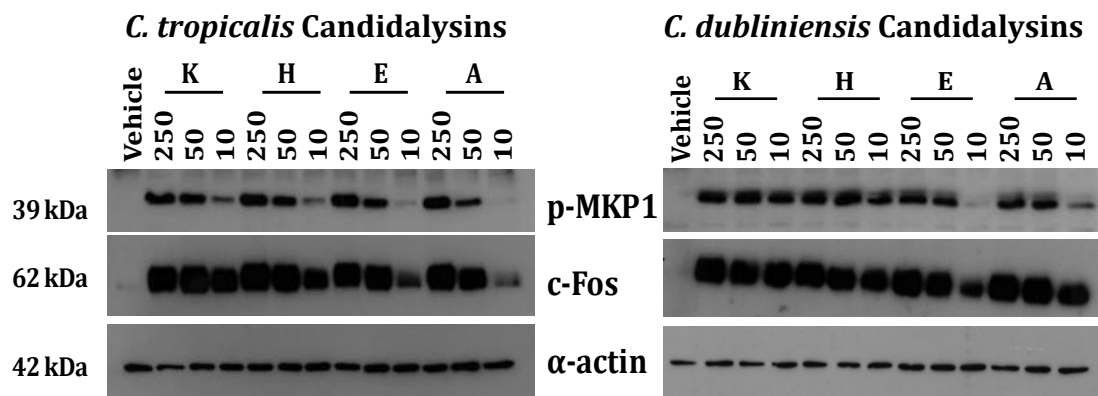


Figura/Figure 4.45 Quantification of lactate dehydrogenase (LDH) activity in the culture supernatant of TR146 epithelial cells to estimate the damage induced by *C. dubliniensis* Candidalysin variants (K, H, E and A). The vehicle bar represents the control experimental conditions with no Candidalysin. Bars represent the mean value of three biological replicates \pm S.E.M. Statistical significance ** $P < 0.01$, *** $P < 0.001$.

4.7.2 c-Fos and MKP-1 induction by Candidalysins of *Candida tropicalis* and *Candida dubliniensis*

We studied c-Fos and p-MKP1 proteins expression of TR146 epithelial cells monolayers after being incubated with *C. tropicalis* or *C. dubliniensis* Candidalysin peptides K, H, E and A.

The four Candidalysin variants of both species of *Candida* induced a strong and dose dependent expression of c-Fos and p-MKP1 compared with the vehicle control. Notably, peptides E and A of both species at their lowest concentrations (10 µg/ml) caused the weakest expression of p-MKP1 when compared with their correspondent K and H variants (Figura/Figure 4.46).



Figura/Figure 4.46 Induction of c-Fos and p-MKP1 in TR146 epithelial cells after 2 h of incubation with Candidalysins variants (K, H, E and A) of *C. tropicalis* (left panel) and *C. dubliniensis* (right panel) at 250, 50 and 10 µg/ml. Vehicle lane represent the basal expression of untreated cells. Expression of α -actin is shown as a reference constitutive protein control. Data is representative of three biological replicates.

4.7.3 Production of cytokines in oral epithelial cells induced by *Candida dubliniensis* and *Candida tropicalis* Candidalysins

We studied the production of several cytokines induced by the presence of the two Candidalysin variants with positively charged amino acids (K and H) of *C. dubliniensis* and *C. tropicalis*.

As we can see in Figura/Figure 4.47 both peptides induced the production of cytokines, in the presence of *C. dubliniensis* Candidalysin variants. The maximum response of G-CSF, GM-CSF, IL-1 β and IL-6 production was reached in the presence of Candidalysin-K and H 50 µg/ml. Both peptides at low concentration (10 µg/ml) increased the production of these cytokines with reference to the control vehicle assay. The results were statistically significant at concentrations ≥ 10 µg/ml. The peptide H at 250 µg/ml caused a statistically significant GM-CSF and G-CSF production when compared with the peptide K.

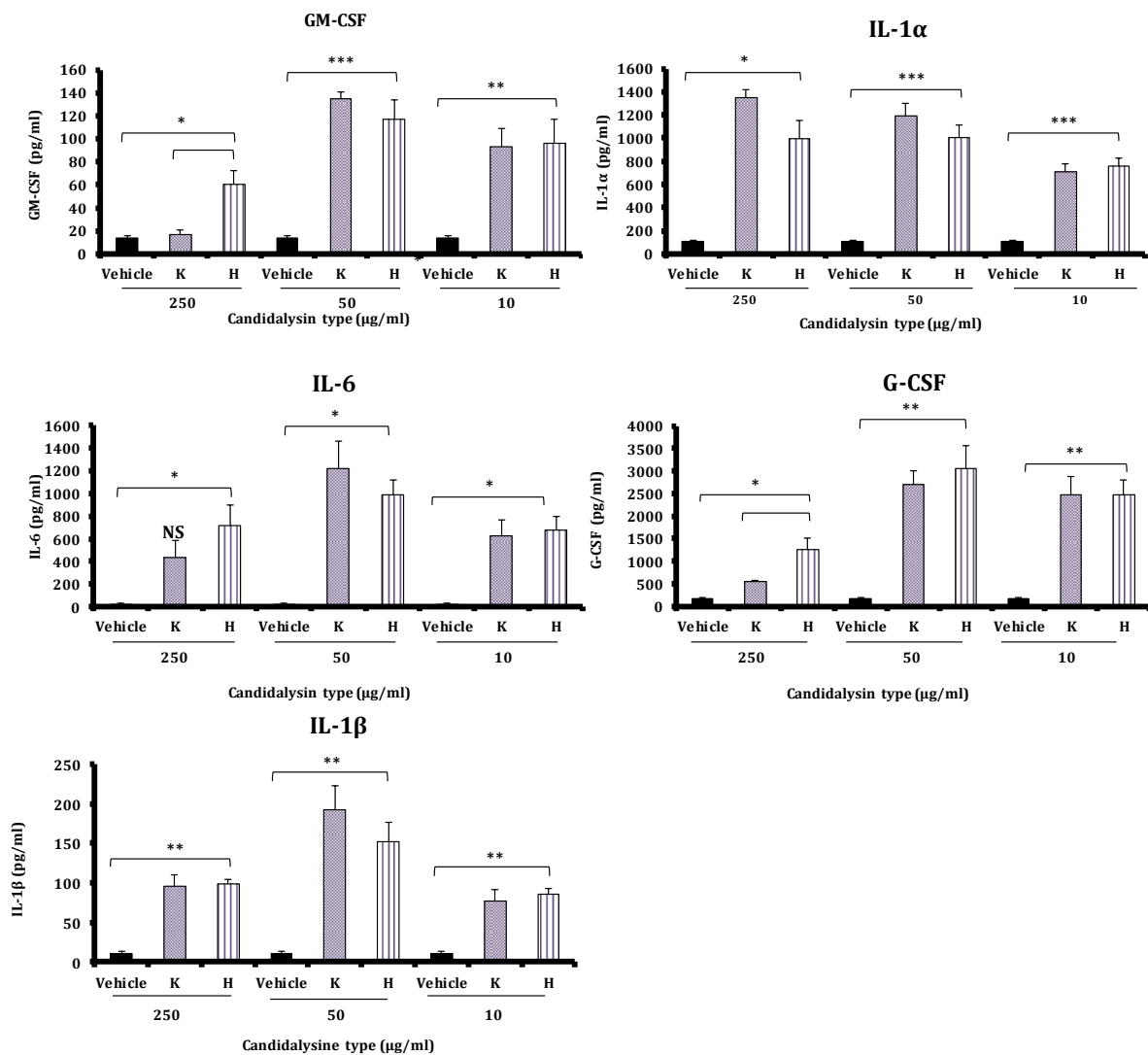
IL-1 α was produced in a dose-dependent manner in the presence of increasing concentrations of Candidalysins. The maximum response of IL-1 α production was reached in the presence of Candidalysin-K and H at 250 µg/ml. The results were statistically significant at concentrations ≥ 10 µg/ml.

As we can see in Figura/Figure 4.48 both peptide variants of *C. tropicalis* induced the production of cytokines as well. IL-1 α , IL-6 and GM-CSF were produced in a dose-dependent manner in the presence of increasing concentrations of Candidalysins. The maximum response was reached at 250 µg/ml, with the exception of Candidalysin-K for

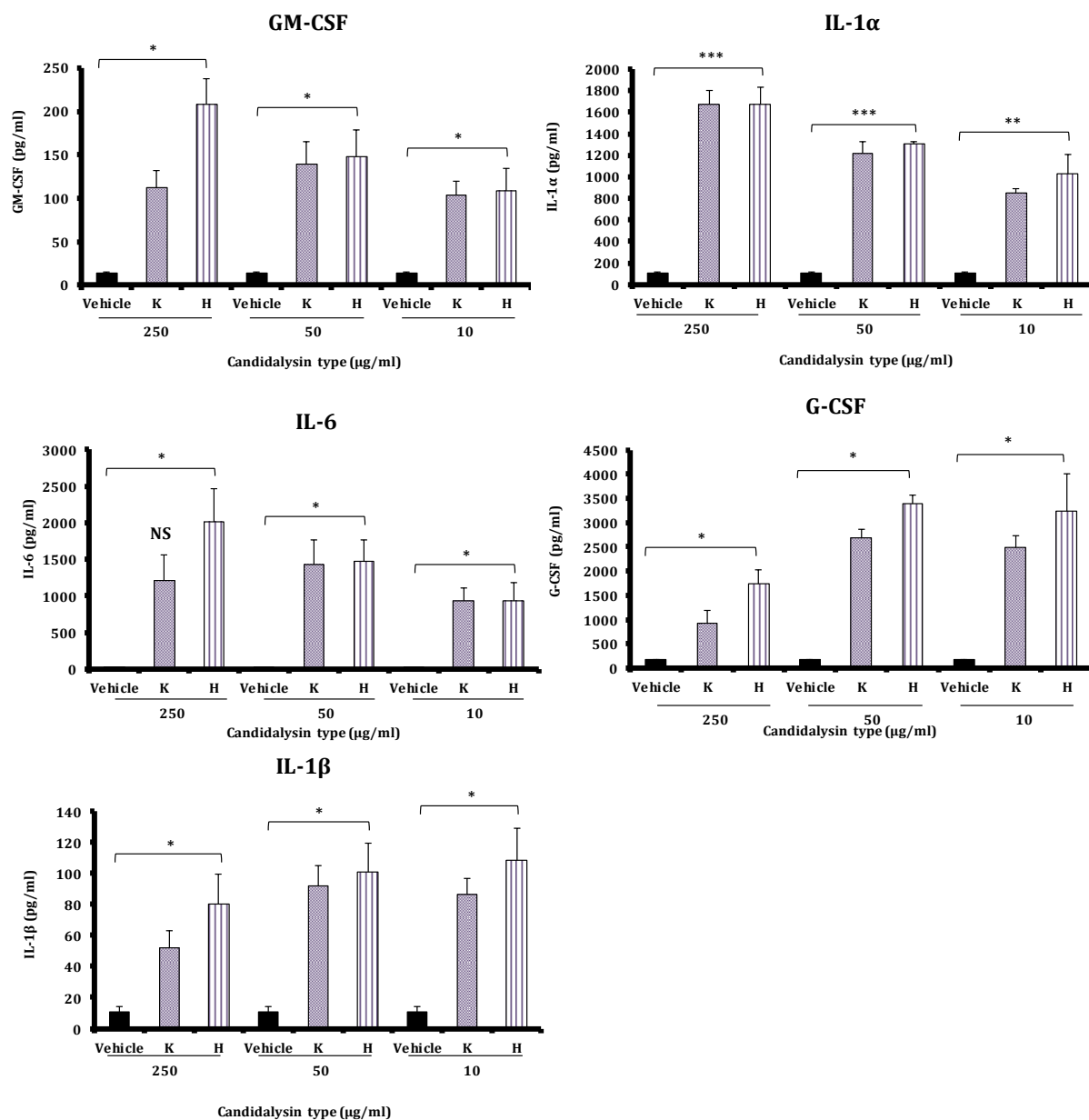


IL-6 and GM-CSF, that reached the maximum at 50 $\mu\text{g/ml}$. The increase was statistically significant at concentrations of Candidalysins ≥ 10 $\mu\text{g/ml}$.

On the other side, the response of G-CSF and IL-1 β production reached maximum values in the presence of Candidalysin-K ≤ 50 $\mu\text{g/ml}$, and dropped down at 250 $\mu\text{g/ml}$. The results were statistically significant at concentrations ≤ 250 $\mu\text{g/ml}$.



Figura/Figure 4.47 Secretion of GM-CSF, IL-1 α , IL-6, G-CSF and IL-1 β by TR146 epithelial cells line after incubation with Candidalysins K and H of *C. dubliniensis* for 24h. Bars represent the mean value of three biological replicates \pm S.E.M. Statistical significance * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.



Figura/Figure 4.48 Secretion of GM-CSF, IL-1α, IL-6, G-CSF and IL-1β by TR146 epithelial cells line after incubation with Candidalysins K and H of *C. tropicalis* for 24h. Bars represent the mean value of three biological replicates ± S.E.M. Statistical significance * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

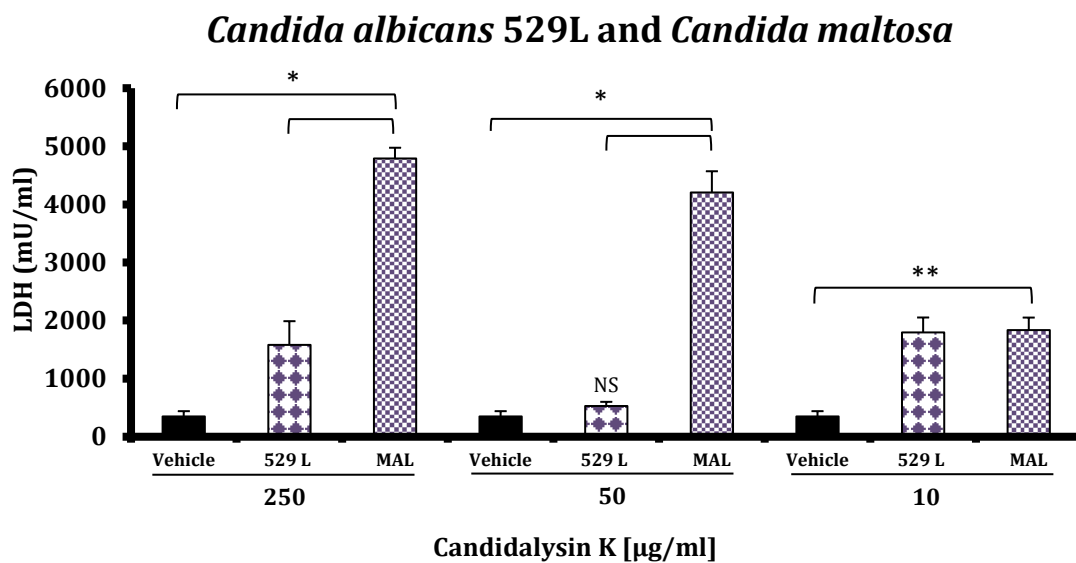
4.8 Effect of Candidalysin of *Candida albicans* 529L and *Candida maltosa* on TR146 epithelial cells line

C. albicans 529L is a clinical strain obtained from a patient colonized by this yeast (Rahman, Mistry, Thavaraj, Challacombe, & Naglik, 2007), and only produces pseudo-hyphae in the presence of epithelial cells (Moyes et al., 2010). On the other hand, *C. maltosa* is unable to produce hyphae, and currently there is no evidence that this species could be a human pathogen. *C. maltosa* Candidalysin sequence shares near 60% homology to that of *C. albicans* SC5314. Consequently, both strains may contribute additional information on the activity characterization of *C. albicans* Candidalysin.



4.8.1 Damage induction of *Candida albicans* 529L and *Candida maltosa* Candidalysins

Candidalysin variant K of *C. maltosa* induced a strong dose dependent cell damage estimated as LDH activity of TR146 epithelial cells, and the effect was statistically significant at concentrations ≥ 10 $\mu\text{g/ml}$, when compared with the non-peptide control (vehicle). Meanwhile, the peptide K of *C. albicans* 529L unexpectedly produced the maximum damage at 10 $\mu\text{g/ml}$ (Figura/Figure 4.49). We observed statistical difference comparing *C. albicans* 529L Candidalysin with *C. maltosa* Candidalysin at concentration ≥ 50 $\mu\text{g/ml}$.



Figura/Figure 4.49 Quantification of lactate dehydrogenase (LDH) activity in the culture supernatant of TR146 epithelial cells to estimate the damage induced by *C. albicans* 529L and *C. maltosa* Candidalysin K variant. The vehicle bar represents the control experimental conditions with no Candidalysin. Bars represent the mean value of three biological replicates \pm S.E.M. Statistical significance * $P < 0.05$, ** $P < 0.01$.

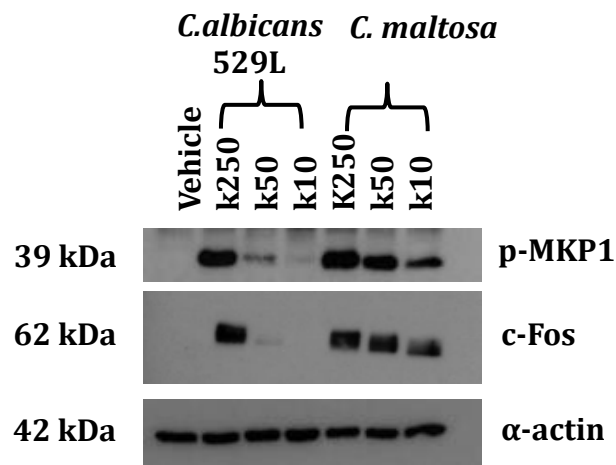
4.8.2 c-Fos and MKP-1 induction by Candidalysins of *Candida albicans* 529L and *Candida maltosa*

We studied c-Fos and p-MKP1 proteins expression of TR146 epithelial cells monolayers incubated with *C. albicans* 529L or *C. maltosa* Candidalysin peptide K.

Both Candidalysins induced a strong and dose dependent expression of c-Fos and p-MKP1 compared with the vehicle control. Notably, the lowest concentrations (50 and 10 $\mu\text{g/ml}$) of *C. albicans* 529L Candidalysin caused a weak expression of p-MKP1 and



c-Fos when compared with the correspondent K variant of *C. maltosa* (Figura/Figure 4.50).



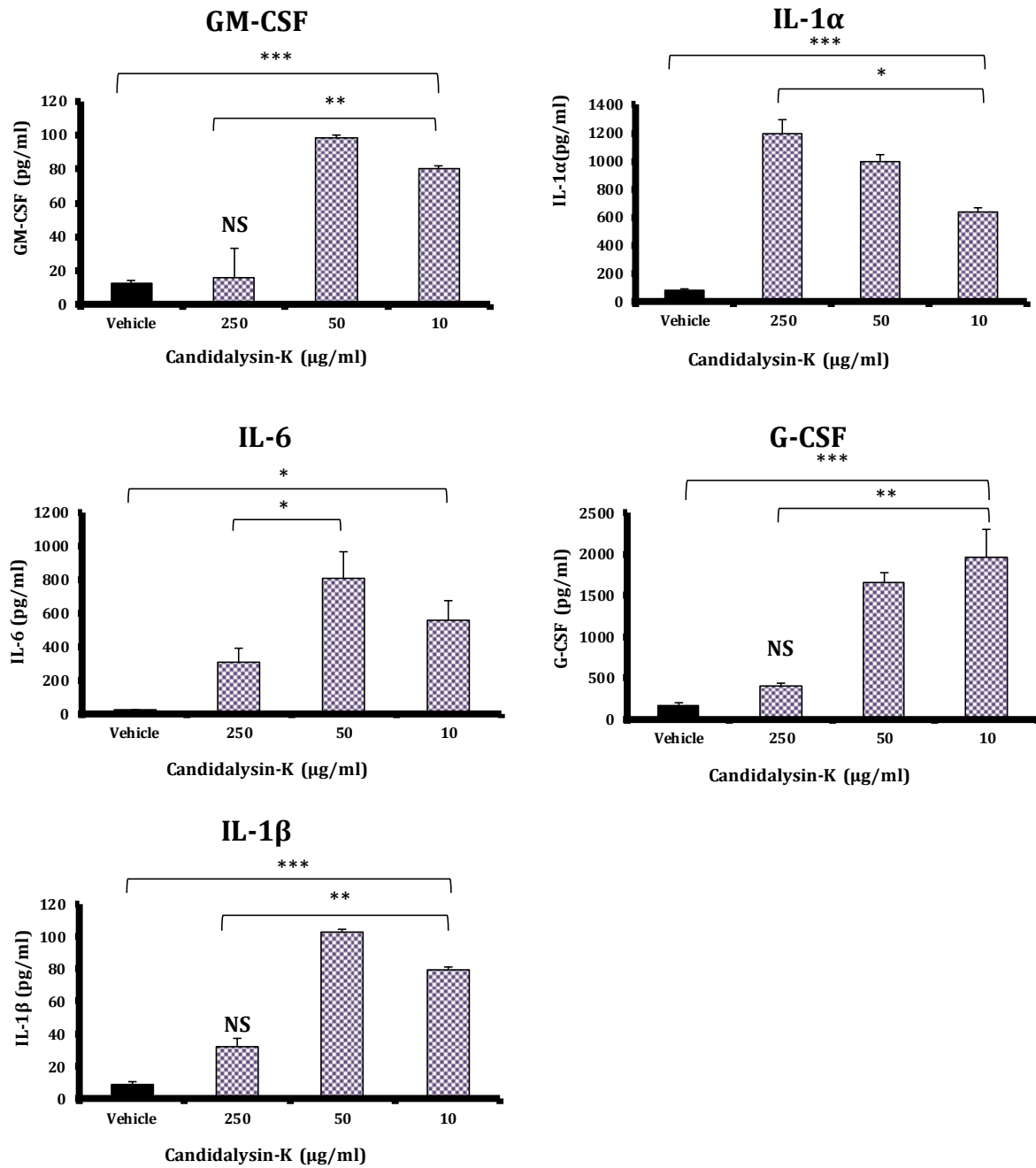
Figura/Figure 4.50 Induction of c-Fos and p-MKP1 after 2 h of incubation TR146 epithelial cells with Candidalysin-k variant of *C. albicans* 529L (left panel) and *C. maltosa* (right panel) at 250, 50 and 10 µg/ml. Vehicle lane represents the basal expression of untreated cells. Expression of α -actin is shown as a reference constitutive protein control. Data is representative of three biological replicates.

4.8.3 Cytokines production in oral epithelial cells induced by *Candida albicans* 529L and *Candida maltosa* Candidalysins

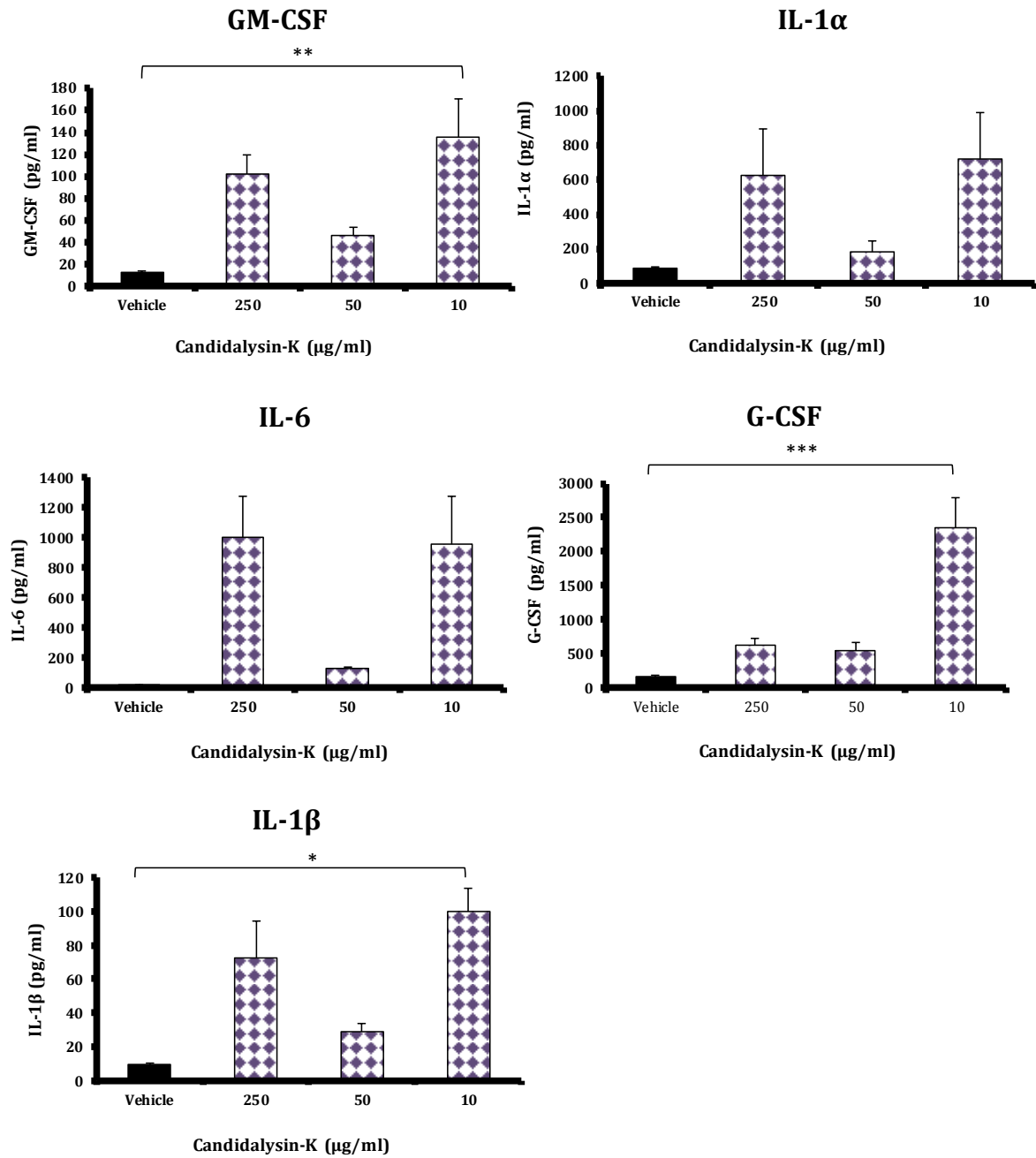
As we can see in Figura/Figure 4.51, *C. maltosa* Candidalysin induced the production of IL 1 α in a dose-dependent manner. The maximum response was reached in the presence of Candidalysin-K 250 µg/ml, and the results were statistically significant at concentrations ≥ 10 µg/ml.

On the other hand, the maximum response of GM-CSF, IL-1 β and IL-6 production was reached in the presence of Candidalysin-K 50 µg/ml or 10 µg/ml for G-CSF. The results were statistically significant at concentrations ≥ 10 µg/ml.

In contrast, *C. albicans* 529L Candidalysin showed the same unexpected trend as revealed in the LDH assay. The maximum production of cytokines was observed at 10 µg/ml followed by 250 µg/ml (Figura/Figure 4.52).

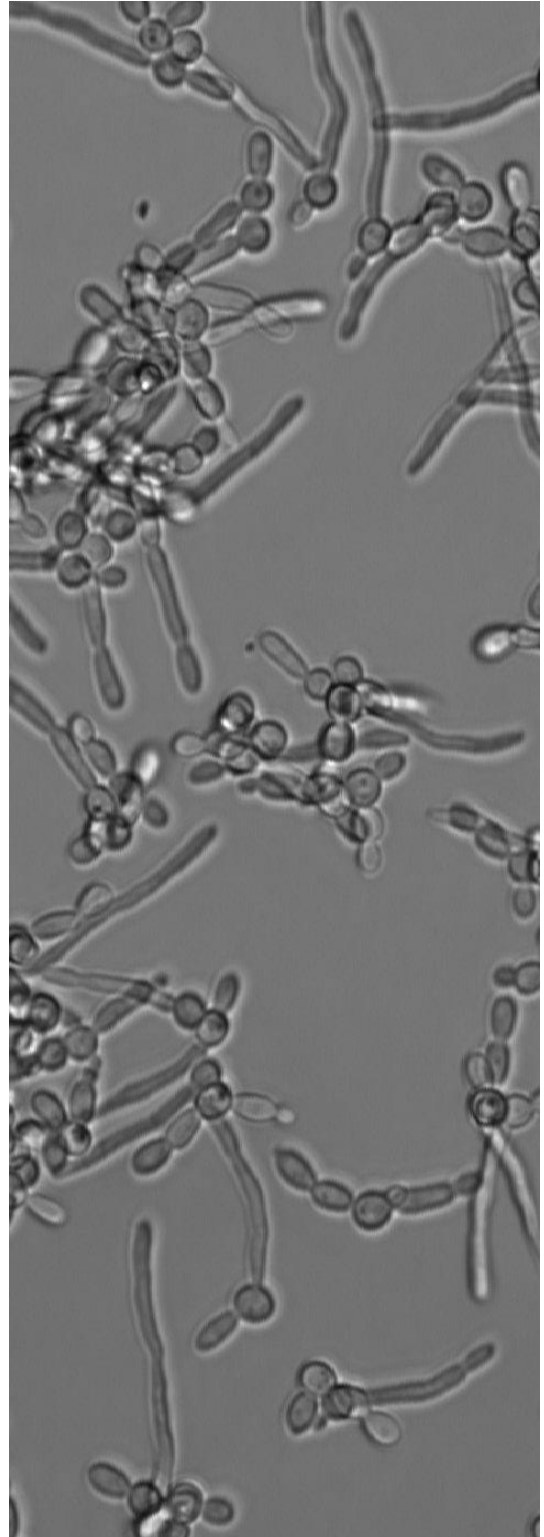


Figura/Figure 4.51 Secretion of GM-CSF, IL-1 α , IL-6, G-CSF and IL-1 β by TR146 epithelial cells line after incubation with Candidalysin K of *C. maltosa* for 24h. Bars represent the mean value of three biological replicates \pm S.E.M. Statistical significance * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.



Figure/Figure 4.52 Secretion of GM-CSF, IL-1α, IL-6, G-CSF and IL-1β by TR146 epithelial cells line after incubation with Candidalysin K of *C. albicans* 529L for 24h. Bars represent the mean value of three biological replicates ± S.E.M. Statistical significance * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

5 Discussion





5

La candidiasis invasora (CI) afecta a más de 250.000 personas en todo el mundo y cada año genera más de 50,000 muertes (Kullberg & Arendrup, 2015). En las últimas décadas, la distribución de las especies aisladas del género *Candida* está sufriendo variaciones en función del área geográfica. Las infecciones provocadas por *C. albicans*, representan menos del 50% de todos los aislamientos causados por hongos del género *Candida* (Arendrup, 2010; Cleveland et al., 2015; Guinea, 2014; Kullberg & Arendrup, 2015; Lamoth, Lockhart, Berkow, & Calandra, 2018). *Candida glabrata* ocupa la segunda posición en el norte de Europa, Estados Unidos y Canadá, mientras que *Candida parapsilosis* ocupa esta misma posición en el sur de Europa, Asia y América del Sur (Kullberg & Arendrup, 2015; Lamoth et al., 2018).

La dificultad diagnóstica de la CI en estadios tempranos de la enfermedad debido a la ausencia de síntomas específicos y a la baja sensibilidad del hemocultivo, considerada técnica de referencia cuyos valores oscilan entre el 17 y el 45% (Arendrup, 2010; Fortun et al., 2014; Nguyen et al., 2012), implican un gran retraso en el tratamiento, lo que en parte explica la alta mortalidad asociada a esta enfermedad. Además, en las últimas décadas, el tratamiento de las infecciones fúngicas invasoras está en constante variación debido a la aparición de cepas cada vez más resistentes (Moragues et al., 2014). Todos estos factores hacen necesaria la búsqueda de vías alternativas para tratar y prevenir la CI.

La integridad del sistema inmune es fundamental no solamente para mantener un equilibrio en las superficies de las mucosas sino, sobre todo, para confinar *Candida* en un estado de comensalismo (Mayer et al., 2013), siendo *Candida* un comensal habitual de piel y mucosas en individuos sanos. En este sentido es indispensable que



nuestro sistema inmunológico sea capaz de discriminar la fase comensal de la invasora y por lo tanto patógena del hongo (Eberl, 2010; Sampaio & Pais, 2017).

Basándonos en estas premisas, nuestro grupo de investigación ha desarrollado una técnica de inmunofluorescencia indirecta (IFI) para el diagnóstico de la CI. La detección en suero de anticuerpos que reaccionan frente a componentes superficiales de la pared de los tubos germinales de *C. albicans* (CAGTA) permite diferenciar entre colonización e invasión ya que el desarrollo de tubos germinales está asociado a la fase invasora (García-Ruiz et al., 1997; Iruretagoyena et al., 2006; Moragues et al., 2004; Quindós, Pontón, Cisterna, & Mackenzie, 1990). El desarrollo de CAGTA también se ha relacionado con el pronóstico de la enfermedad, ya que pacientes con CI ingresados en UCI y que mostraban títulos crecientes de CAGTA alcanzaban mayores tasas de supervivencia (Huertas et al., 2016). Pitarch y colaboradores (Pitarch, Nombela, & Gil, 2011) también registraron un mejor pronóstico en pacientes con títulos elevados de anticuerpos anti-Met-6, anti-Hsp90 y anti-Pgk1. En esta misma línea, en un modelo murino de CI, Huertas y colaboradores (Huertas et al., 2016) observaron que los ratones infectados con *C. albicans* y previamente colonizados por el mismo hongo, presentaban tasas de supervivencia mayores con respecto a ratones infectados con *C. albicans* sin colonización previa. Esta observación venía acompañada de títulos elevados de IgG frente a las proteínas Eno-1, Met-6, Hsp70, Pcd11, Pgk1 y Cdc19, muchas de las cuales son reconocidas por los CAGTA según estudios realizados en nuestro laboratorio (Sáez-Rosón et al., 2014).

Con estos antecedentes, un objetivo de este estudio fue evaluar la actividad biológica frente a *Candida albicans* de los anticuerpos desarrollados en un modelo de CI en conejo, y verificar posteriormente dicha actividad en pacientes con CI. Al mismo tiempo nos propusimos caracterizar el patrón de reconocimiento antigénico de los CAGTA mediante el cribado de una genoteca de cDNA de micelios de *C. albicans* en el fago λ ZapII. Por otro lado, se estudió el rol del péptido EceIII, derivado de la proteína Ece1p, específica de la fase filamentosa y por lo tanto invasora del hongo, en la producción de daño a nivel celular y en la activación de la respuesta p-MKP1/c-Fos con la subsiguiente producción de citoquinas.



En este trabajo hemos confirmado que las IgG totales del suero de un modelo animal de CI de conejo reconocen antígenos de la superficie de blastosporas y tubos germinales de *C. albicans* mediante inmunofluorescencia indirecta, y la proporción de IgG específicas en el suero aumentaba en el curso de la infección tras repetidas tandas de inoculación con esta levadura. Estos resultados concuerdan con la evolución de los títulos de IgG presentados en el trabajo de Sáez-Rosón y colaboradores (Sáez-Rosón et al., 2014), donde los títulos CAGTA aumentaron moderadamente a las 2-4 semanas tras la primera inoculación con *C. albicans*, y llegaron a títulos máximos después de la tercera inoculación, estabilizándose en su respuesta. De este modo, se eligieron los sueros más tardíos para obtener un mayor rendimiento en CAGTA para los estudios posteriores. En el modelo de infección por *C. albicans* en conejo, el título de anticuerpos anti-*Candida* de clase IgG aumenta tras el contacto repetido con el hongo en sucesivas inoculaciones.

Algunas de las proteínas reconocidas por los CAGTA ya han sido previamente identificadas por nuestro grupo en extractos de pared, incluyendo entre otras Met-6, Ino1, Eno-1, Adh1, Pgl1 y 14-3-3 (Sáez-Rosón et al., 2014). Dado que los CAGTA reaccionan de forma específica frente a los tubos germinales de *C. albicans*, quisimos estudiar la posible contribución de los anticuerpos específicos en el reconocimiento de algunos de estos antígenos mediante ensayos ELISA. Así, se estudió la detección de anticuerpos contra las proteínas de *C. albicans* 14-3-3, Adh1, Als3-N, Eno-1, Met-6, Hwp1-N y una fracción eluida del extracto de pared de micelios correspondiente a la enolasa (Eno-pared).

Los sueros de los dos conejos infectados reaccionaron de forma paralela con las diferentes proteínas ensayadas a lo largo del proceso de candidiasis invasora inducida, por lo que los comentaremos en conjunto. En el caso de la proteína 14-3-3, se detectaron niveles bajos de anticuerpos específicos cuyo título se mantenía estable a lo largo de la infección. Aunque en nuestro laboratorio se ha podido comprobar que los sueros de pacientes con candidiasis reaccionaban con esta proteína (Sáez-Rosón, 2015; datos no publicados), en el modelo animal los anticuerpos frente a 14-3-3 no mostraron evidencias como responsables del incremento en la respuesta de CAGTA en las fases avanzadas de la infección.



Los anticuerpos anti-Als3-N también mostraron una respuesta discreta con un ligero incremento a lo largo de todo el proceso. A pesar de no existir una relación proporcional con el incremento en el título de CAGTA, diferentes autores han obtenido resultados de protección muy prometedores con la proteína recombinante Als3-N, cuya administración generaba una respuesta inmunológica clave en la estimulación de linfocitos Th1/Th17 en ratones infectados con *C. albicans*, consiguiendo una tasa de supervivencia del 50% frente a la mortalidad de todo el grupo control (Shahid, 2016; Spellberg et al., 2008; Vecchiarelli, Pericolini, Gabrielli, & Pietrella, 2012). En este sentido, se ha desarrollado la vacuna NDV-3 basada en la proteína recombinante Als3p-N, que se encuentra en fase clínica 1; la inyección de 30-300 µg de dicha vacuna en 40 individuos sanos produjo títulos elevados de IgG en suero (Schmidt et al., 2012). Más recientemente, la versión NDV-3A de esta vacuna, enfocada a la candidiasis vulvovaginal, se encuentra en fase clínica 2 (Scorzoni et al., 2017). La administración de una única dosis de NDV-3A en 188 pacientes con candidiasis vulvovaginal recurrente, ha generado una rápida y robusta respuesta inmunológica reduciendo hasta en un 50% la reaparición de *Candida* (NovaDigm Therapeutics, 2016).

Por otro lado, los anticuerpos anti-Hwp1-N, anti-Eno-1 y anti-CW-Eno incrementaron su título de forma continuada, con una importante respuesta tras la segunda inoculación. Estos resultados son comparables con estudios realizados previamente en nuestro grupo y así, la titulación de anticuerpos anti-Hwp1-N en sueros seriados de pacientes con CI mostró una cinética con valores elevados ya en estadios tempranos de la enfermedad (Corouge et al., 2015; Laín et al., 2007a). En relación con su utilización en ensayos de protección, Xin y colaboradores observaron una mayor supervivencia en ratones infectados con *C. albicans* que habían sido inmunizados con Hwp1-N y Eno-1 conjugadas a β -manano; en el caso del conjugado con la Hwp1-N se obtuvo un 80-100% de supervivencia, y un 40-80% con la Eno-1 (Xin, Dziadek, Bundle, & Cutler, 2008). Similarmente, en un modelo animal de ratón, los títulos anti-Eno-1 se observaron tan sólo transcurridos los primeros 6 (He et al., 2015) y 10 días (Huertas et al., 2016) de infección con *C. albicans*. Estos anticuerpos anti-Eno-1 también se han observado en pacientes durante los primeros días de evolución de una candidiasis invasora (Laín et al.,



2007b), y relacionado con esta respuesta también se ha propuesto la detección de anticuerpos anti-Eno-1 como prueba diagnóstica de CI en pacientes tanto inmunocomprometidos como inmunocompetentes (Laín et al., 2007b). Estudios posteriores realizados por Shibasaki y colaboradores mostraron que ratones a los que se administraba por vía oral la proteína recombinante Eno-1 y después eran infectados con *C. albicans*, presentaban un aumento en las tasas de supervivencia, así como elevados títulos de IgG anti-Eno-1 en suero (Shibasaki et al., 2013). Por lo que respecta a la Eno-pared, se correspondería con la Eno-1 que han estudiado recientemente Reyna-Beltrán y colaboradores (Reyna-Beltran et al., 2018). Estos autores han propuesto que este enzima de la pared de *C. albicans* puede presentar actividades enolasa y transglutaminasa (TGasa) localizadas en diferentes locus de su molécula, estando involucrado en el crecimiento, división celular, morfogénesis y protección osmótica del hongo. Por este motivo, la TGasa/Eno podría ser una diana en el diseño de nuevos fármacos para el control de la infección por *C. albicans*.

Finalmente, cabe destacar la respuesta de los anticuerpos anti-Met-6 y anti-Adh1, cuyos títulos aumentaron dramáticamente tras la cuarta inoculación de los conejos con *C. albicans*. Estos datos contrastan con los títulos de anticuerpos registrados por Saéz-Rosón y colaboradores, quienes observaron que los CAGTA se incrementaban de forma continua desde la primera inoculación con *Candida* (Sáez-Rosón et al., 2014). Sin embargo, en estudios previos en nuestro laboratorio (Sáez-Rosón, 2015; datos no publicados) mediante ensayos ELISA, se han detectado títulos elevados y tempranos de anticuerpos anti-Met-6 en pacientes infectados por *Candida*; no se obtuvieron resultados similares con los títulos anti-Adh1. Por otro lado, Xin y colaboradores registraron tasas elevadas de supervivencia del 80-100% en ratones infectados con *C. albicans* e inmunizados con la proteína Met-6 conjugada a β -manano (Xin et al., 2008). Más recientemente, el mismo grupo de investigación estudió la respuesta de ratones neutropénicos vacunados con péptidos de Met-6 y posteriormente infectados con *C. albicans*, registrando un incremento en su tasa de supervivencia respecto al grupo control. De forma paralela, generaron anticuerpos monoclonales frente al péptido Met-6 que se utilizaron para proteger ratones neutropénicos, y observaron tasas de supervivencia entre el 20 y el 60% (Xin, 2016). En relación a la proteína Adh1, Trautwein-Weidner y colaboradores, vieron como



células dendríticas activadas con el péptido Adh1₁₂₆₋₁₄₀ eran capaces de estimular una respuesta Th17 en hibridomas de células T, en relación con un modelo de candidiasis orofaríngea en ratones (Trautwein-Weidner et al., 2015).

En esta primera fase del trabajo, podemos concluir que la actividad de los anticuerpos anti-Adh1 y anti-Met-6 podrían encontrarse entre los principales responsables de la mayor reacción de inmunofluorescencia frente a *C. albicans* en los sueros de un modelo de candidiasis invasora en conejos en estadios más avanzados de la infección; mientras tanto, la respuesta más temprana de los anticuerpos anti-Eno-1, anti-eno-pared y anti-Hwp1-N, hace de sus correspondiente proteínas (Eno-1, Eno-pared y Hwp1-N) importantes inmunógenos durante las primeras fases de la infección. Estos hallazgos refuerzan la candidatura de estas proteínas como posibles vacunas frente a la candidiasis invasora ya que inducen títulos elevados de anticuerpos, de aparición temprana en el proceso infeccioso.

Con el fin de ampliar la lista de los antígenos reconocidos por los CAGTA, se realizó el cribado de una genoteca de expresión en el fago λ ZapII obtenida a partir de cDNA de *C. albicans* creciendo en fase micelial. De forma análoga a los estudios proteómicos descritos previamente, las proteínas o fragmentos peptídicos seleccionados pueden ser la base para el desarrollo de técnicas de diagnóstico específicas para la CI, o bien ser utilizados como inmunógenos protectores frente a la CI (Chikaev et al., 2015; Riemer et al., 2005; Wierzbicki et al., 2008).

El tamaño del genoma de *Candida* se estima en $1,4 \times 10^4$ Kb, y la genoteca utilizada en este trabajo presentaba un porcentaje de recombinación del 97% con un tamaño medio de inserto de 2,0 Kb. Con estas premisas se decidió plaquear 60.000 fagos, que se corresponden a 8 veces el genoma de *Candida*, pudiendo de esta forma asegurarnos la representatividad del escrutinio. De acuerdo con Sambrook y Russell (Sambrook & Russel, 2001) los epítomos reconocidos por los anticuerpos, en nuestro caso los CAGTA, esperamos que sean secuenciales. La expresión en fagos de la genoteca de cDNA de *Candida* nos permitió localizar 59 clones que reaccionaban con los CAGTA, de los cuales se seleccionaron 5 clones que expresaban proteínas o fragmentos peptídicos de *C. albicans* con un patrón de lectura en fase. Los cinco clones reaccionaban con los CAGTA tanto del modelo de CI de conejo, como de



pacientes con CI. La comparación con las bases de datos permitió su identificación como: fosfoglucoisomerasa-1 (glucose-6-p-isomerase, PGI1), hyphal wall protein (Hwp1), ácido graso sintasa (fatty acid synthase, Fas1), proteína de respuesta al pH (pH-responsive protein 1, Phr1), y actina (Act1).

Todas estas proteínas se encuentran en distintos compartimentos celulares de *C. albicans* y sus funciones están de alguna forma relacionadas con la formación de hifas. Además, se ha visto que están sobreexpresadas en la formación de biopelículas o directamente involucradas en la pared celular del hongo.

La glucosa-6-fosfato isomerasa (PGI1), correspondiente al clon A2, ha sido identificada previamente por Pitarch y colaboradores mediante un patrón de reconocimiento antigénico utilizando sueros de pacientes con CI (Pitarch et al., 2004), y está involucrada en el metabolismo de los carbohidratos. Marchais y colaboradores relacionaron su incremento con una mejor adherencia a placas de poliestireno y la formación de tubos germinales (Marchais et al., 2005; Tronchin, Bouchara, Robert, & Senet, 1988)

El clon D1 se corresponde a la proteína Hwp1. Esta proteína, como ya se ha comentado anteriormente, se expresa únicamente en la fase micelial de *C. albicans* y se localiza en la pared de los micelios. Se ha visto que su presencia es necesaria para la formación de biopelículas tanto *in vivo* como *in vitro* (Mishra, Aier, Varadwaj, & Misra 2018; Nobile et al., 2006). Se han observado elevados niveles de expresión del gen *Hwp1* durante la fase de filamentación de *Candida* (Creger & Blankenship, 2018; Naglik et al., 2006). Esta proteína también se ha visto involucrada en fenómenos de adaptación al estrés, así como en la patogenicidad de *Candida* (Mishra et al., 2018)

El clon H1, que se corresponde al gen *FAS1*, codifica para la subunidad β del enzima ácido graso sintasa (Fas1). Esta enzima se ha visto involucrada en la expresión del factor de transcripción EFG1 directamente relacionado con la formación de hifas y de la pared celular de *C. albicans* (Singh, Sinha, & Sadhale, 2005).

El clon N1 codifica para la proteína Phr1, que ejerce como un regulador de pH estrechamente relacionado con la adhesión e invasión del hongo. Cepas mutantes



knock-out de este gen, presentan grandes dificultades en la penetración e invasión de las primeras capas del epitelio, así como para el crecimiento micelial. Calderón y colaboradores vieron que las hifas de cepas mutantes para este gen eran más cortas y presentaban distorsiones evidentes comparándolas con cepas salvajes (Calderon et al., 2010).

El clon R1 se corresponde a la proteína Act1, una beta-actina que forma parte del citoesqueleto de *C. albicans*. Pertenece a un grupo de genes altamente conservados involucrados en distintas actividades como la motilidad celular. Hasta el momento no se han encontrado diferencias en su expresión entre las fases levaduriforme y micelial, presentando un nivel de expresión muy estable (Nailis, Coenye, Van Nieuwerburgh, Deforce, & Nelis, 2006)

El reconocimiento de estas proteínas por los CAGTA nos llevó a analizar su antigenicidad, comprobando que no existe homología entre los epítomos inmunogénicos de dichas proteínas. De este modo, parece que las diferentes proteínas han generado diferentes líneas de respuesta IgG específica.

Sentandreu y colaboradores construyeron genotecas de expresión en el fago λ gt11 a partir de ambos morfotipos de *C. albicans*. Mediante la utilización de anticuerpos policlonales producidos en conejos inmunizados con componentes purificados de la pared de *Candida* (tanto de micelio como de levadura), identificaron dos genes que se corresponden a la Enolasa de pared y otra proteína específica de la fase micelial denominada Csp37 (Sentandreu, Elorza, Valentin, Sentandreu, & Gozalbo, 1995; Sentandreu et al., 1997).

En un estudio de naturaleza similar, el cribado de una genoteca de expresión de tubos germinales de *C. albicans* generada en *E. coli*, con IgG de individuos con VIH y candidiasis permitió identificar 12 clones, los cuales se correspondían con Hwp1, CST20, CPP1, RBF1, PTS1/PTH1, CDC24, PCT1, LPD1, NOT5 (orf6.6343), orf6.7858, y la mayoría están relacionadas con la formación de hifas, biopelículas y virulencia (Cheng et al., 2003; Pitarch et al., 2008). Las proteínas identificadas en nuestro trabajo coinciden en algunos casos con lo descrito por otros autores (Hwp1, enolasa), sin embargo, en el caso de PGI1, Fas1 y Phr1 esta sería la primera mención como agentes inmunogénicos en un modelo animal de candidiasis invasora. Además,



se confirmó que los sueros de pacientes con CI también reaccionaban frente a los clones previamente seleccionados con los CAGTA del modelo de candidiasis en conejo.

Por lo tanto, la fracción de suero enriquecida en CAGTA de un modelo de CI de conejo incluye anticuerpos de distintas especificidades frente a antígenos cuya expresión está relacionada con la fase micelial de *C. albicans*. Además de la Hwp1 que ya conocíamos, se incluyen Act1, Fas1, PGI1 y Phr1.

Los pacientes con candidiasis invasora también desarrollan una respuesta de anticuerpos específica frente a las proteínas expresadas por los clones seleccionados en el modelo de candidiasis en conejo.

Dado que los pacientes, con CI e ingresados en UCI, que han desarrollado CAGTA presentaban un mejor pronóstico (Zaragoza et al., 2009a), quisimos determinar la actividad que presentaban los sueros inmunes que contenían los CAGTA frente a células de *C. albicans* creciendo tanto en suspensión como formando biopelículas.

Tradicionalmente, la viabilidad de cultivos celulares de *C. albicans* se ha determinado mediante el recuento de colonias. El problema de este método es que no informa acerca de la capacidad metabólica de las células de *Candida*, la incapacidad de algunas de ellas para dividirse activamente y, además, requiere tiempos prolongados. Además, *C. albicans* es una cepa que filameta a 37°C, por lo que los agregados entre células y filamentos pueden producir errores importantes en el recuento de colonias. Por esto motivo se decidió realizar junto con el recuento de colonias, el ensayo colorimétrico XTT basado en sales de tetrazolio, con el que se evalúa la actividad metabólica celular.

Nuestros resultados mostraron correlación entre el ensayo colorimétrico y el recuento de UFC a la hora de evaluar la actividad de los anticuerpos tanto en el modelo animal de CI como con suero de pacientes.

En general, en este trabajo se encontró una relación dosis-dependiente entre la concentración de anticuerpos y la actividad antifúngica frente a *C. albicans*. La fracción total de IgG tanto en el modelo animal como en pacientes con CI reducía la actividad metabólica y la viabilidad del hongo. La reducción de la actividad



metabólica del hongo estaba directamente relacionada con los títulos de anticuerpos anti-micelio en suero.

Con el fin de delimitar la contribución de las distintas fracciones de IgG, se analizó el efecto de los anticuerpos anti-BI y los de la fracción enriquecida en CAGTA (CAGTA-enr) por separado. La fracción anti-BL de suero inmune de conejo, mayoritariamente anticuerpos anti-manano, no mostró ningún efecto significativo en la reducción de la actividad metabólica de *C. albicans*. Esta observación contrasta con los estudios llevados a cabo por Zhang y colaboradores (Zhang, Lupan, & Kozel, 1997; Zhang & Kozel, 1998) y por Kozel y colaboradores, quienes mostraron que los anticuerpos anti-manano reducían la actividad metabólica de *C. albicans*, si bien en sus experimentos usaban suero completo en el que estaba presente el sistema del complemento (Kozel, MacGill, Percival, & Zhou, 2004).

En cambio, la fracción enriquecida en CAGTA afectó no solo a la actividad metabólica, sino también a la viabilidad de las levaduras tratadas, aunque su efecto fue inferior en comparación a la fracción total de IgG. Existen estudios de inhibición de la actividad metabólica a partir de modelos animales de Candidiasis con resultados muy similares a los nuestros. Fujibayashi y colaboradores, produjeron anticuerpos policlonales a partir de huevos de gallinas infectadas con *C. albicans* SC5314 y observaron una reducción del crecimiento y de la actividad metabólica del hongo en contacto con estos anticuerpos (Fujibayashi et al., 2009).

De la misma forma, Brena y colaboradores observaron que el anticuerpo monoclonal C7 producido a partir de ratones BALB/c infectados con blastosporas de *C. albicans* (Moragues et al., 2003) causaba reducción en la adhesión de *C. albicans*, y mostraba actividad fungicida (Brena et al., 2007).

El recuento de unidades formadoras de colonias (UFC) ha respaldado los resultados obtenidos mediante el ensayo colorimétrico, aunque estos podrían estar influenciados por la formación de filamentos y agregados en las placas de agar Sabouraud. Capodicasa y colaboradores obtuvieron resultados en la misma línea con un anticuerpo recombinante derivado del monoclonal anti- β -glucano (mAb 2G8), capaz de reducir el crecimiento e inhibir *C. albicans* tanto *in vitro*, como *in vivo* con una reducción significativa de UFC en riñón (Capodicasa et al., 2011).



El análisis morfológico del cultivo en presencia de CAGTA reveló que las levaduras tratadas mostraban inhibición del crecimiento en función de la concentración de IgG. Aunque la aparición de tubos germinales resulta paradójica, la microscopía electrónica reveló que la superficie de las levaduras no estaba alterada por la presencia de CAGTA, mientras que los tubos germinales presentaban protuberancias en la superficie de la pared celular. Como apoyo a esta observación la tinción vital reveló que estos últimos no eran viables.

Nuestro grupo de investigación, utilizando el anticuerpo monoclonal C7 o el péptido CDR-H2 derivado de este anticuerpo, registró mediante microscopía electrónica alteraciones de naturaleza similar en células levaduriformes de *Candida* (Cabezas, 2011; datos no publicados). Estas alteraciones en la pared celular pueden reducir la viabilidad del hongo, ya que podrían afectar al tráfico de nutrientes a través de la pared celular, a la estabilidad osmótica y a la organización final de la misma pared. Otros autores han registrado también alteraciones severas en la pared celular de *Candida*, e incluso de la membrana citoplasmática, en presencia de un péptido de guindilla (Ribeiro et al., 2007). Hashemi y colaboradores (Hashemi et al., 2018) observaron alteraciones morfológicas, de naturaleza similar a las registradas en este trabajo en la pared celular de *C. auris* tratada con el antifúngico ceragenina CSA-131. Estos aspectos concuerdan con el efecto fungicida de los CAGTA sobre *C. albicans*, respaldado por las tinciones fluorescentes y las imágenes obtenidas mediante microscopía electrónica. Y por otro lado, dado que los CAGTA se obtienen por elución a partir de su reacción con la pared de los tubos germinales de *C. albicans*, cabe esperar que no actúen sobre el hongo en fase levaduriforme.

Cabe destacar que la IgG neutra, incluida como control en nuestro estudio, redujo inesperadamente la actividad metabólica de *C. albicans*. Un efecto similar fue descrito por Rodier et al (Rodier, Imbert, Kauffmann-Lacroix, Daniault, & Jacquemin, 2003) con una IgG neutra irrelevante. A pesar de ello, en nuestro estudio esta reducción nunca alcanzó los mismos porcentajes que la fracción total de IgG, así como de la fracción enriquecida en CAGTA. Además, no se observó relación dosis-dependiente entre concentración de anticuerpo y la actividad antifúngica frente *Candida*. A esto habría que añadir que el recuento de unidades formadoras de colonias, tampoco se vió afectado por la IgG neutra en comparación con el grupo control sin tratamiento.



Basándonos en estos resultados, podemos asumir que las IgG de los sueros inmunes de conejos con CI reducen la actividad metabólica de las levaduras de *C. albicans in vitro*. Mientras que las IgG que reaccionan con la superficie de las levaduras no ejercen ningún efecto observable, la fracción IgG del suero enriquecida en CAGTA soporta la mayor actividad y afecta a la estructura de la pared de los tubos germinales. Además, los CAGTA purificados a partir del modelo animal de CI en conejo muestran actividad fungicida frente a los tubos germinales de *C. albicans*.

En el caso de sueros de pacientes con CI se analizó la actividad inhibitoria en función de los títulos y de la especie de *Candida* que había generado la infección. Los sueros de pacientes con CI reprodujeron los resultados obtenidos con el modelo animal. La fracción enriquecida en CAGTA de sueros de pacientes con una infección invasora por *C. albicans* mostró capacidad para inhibir la actividad metabólica del hongo, siendo más acusada en aquellos pacientes con títulos anti-micelio elevados. Más aún, los anticuerpos CAGTA purificados a partir de sueros de pacientes con CI mostraron un efecto fungicida similar al del modelo animal, registrándose también alteraciones en la superficie de los tubos germinales. Clancy y colaboradores observaron que títulos elevados de determinadas IgG en suero (anti-SET1, anti-ENO-1, anti-MUC1-2, y anti-PGK1-2), además de ser útiles para el diagnóstico de una CI, son un posible marcador predictivo durante la fase de convalecencia de los pacientes afectados por una candidemia (Clancy et al., 2008). Por su parte, Zaragoza y colaboradores (Zaragoza et al., 2009a) registraron observaciones similares con los títulos de CAGTA, lo cual se ve apoyado por el efecto anti-*Candida* que hemos podido registrar *in vitro* para estos anticuerpos.

De un modo similar a lo registrado en el modelo animal, los sueros de pacientes con una CI causada por *C. albicans* reducen la actividad metabólica de las levaduras de *Candida in vitro*. Así mismo, la principal actividad se observa en la fracción CAGTA-enr de pacientes con candidemia, la cual afecta a la estructura de la pared de los tubos germinales. Los CAGTA purificados a partir de pacientes con CI tienen capacidad fungicida.

En cambio, el análisis de la actividad antifúngica de los anticuerpos de pacientes infectados por especies de *Candida no-albicans* mostró una menor capacidad media



para inhibir la actividad metabólica o la viabilidad de *C. albicans*. En estos casos, las IgG de pacientes infectados por *C. parapsilosis* fueron las de mayor efecto sobre la actividad metabólica de *C. albicans*, junto con un caso de co-infección por *C. glabrata/C. tropicalis*; por el contrario, las IgG provenientes de pacientes infectados con *C. tropicalis* fueron las menos activas.

Esta actividad podría atribuirse a un fenómeno de reacción cruzada previamente descrito en ensayos de detección de CAGTA mediante inmunofluorescencia (Moragues et al., 2004), así como en ensayos inmunoenzimáticos (Laín et al., 2007a; Li et al., 2013; Pitarch, Nombela, & Gil, 2007). Laín y colaboradores observaron que sueros de pacientes infectados por *C. parapsilosis*, *C. tropicalis*, *C. utilis*, *C. glabrata* o *C. dubliniensis* reaccionaban con la proteína recombinante Hwp1-N de *C. albicans*, y lo atribuían a la existencia de epítomos comunes entre la Hwp1 de *C. albicans* con otras proteínas del género *Candida* (Laín et al., 2007a). De la misma forma, otros autores observaron que los sueros de pacientes infectados con *C. tropicalis*, *C. parapsilosis*, *C. glabrata* o *C. guilliermondii* reaccionaban frente a la proteína recombinante Eno-1 de *C. albicans* (Clancy et al., 2008; Li et al., 2013). Bajo determinadas condiciones, algunas especies de *Candida* no-*albicans* son capaces de expresar antígenos que generarían unos anticuerpos que reaccionarían de forma cruzada con *C. albicans*, como es el caso de *C. tropicalis*, *Candida stellatoidea*, *C. parapsilosis* y *C. guilliermondii* (Bikandi et al., 1998; Laín et al., 2007a). En este mismo sentido, Pitarch y colaboradores (Pitarch et al., 2007) también observaron que la proteína Met-6 de *C. albicans* era reconocida por anticuerpos de pacientes infectados por otras especies de *Candida*.

En su momento, también el anticuerpo monoclonal Mycograb® (Novartis, AG, Basel, Suiza) demostró capacidad para inhibir el crecimiento no solamente de *C. albicans*, sino también de otras especies como *C. glabrata*, *C. krusei*, *C. parapsilosis* o *C. tropicalis* (Matthews et al., 2003). Estos resultados concuerdan con la acción cruzada observada en este proyecto sobre la actividad metabólica y vitalidad de *C. albicans* en contacto con IgG de pacientes infectados con especies de *Candida* no-*albicans*.



Los pacientes infectados por una especie de *Candida no-albicans* generan anticuerpos que muestran reacción cruzada con *C. albicans* reduciendo su actividad metabólica y viabilidad, aunque con menor intensidad.

While there is an increasing evidence of the inhibitory activity of CAGTA against *Candida* planktonic cells, no data are still available on the ability of such Abs to prevent biofilm formation, one of the major virulence factors of *C. albicans*. To the best of our knowledge, this is the first report showing that CAGTA reduce *Candida* biofilm formation. In particular, our data indicate that CAGTA impair biofilm formation mainly at the very early stages, within the first 90 minutes after seeding *C. albicans*; in contrast, CAGTA showed no activity against an already structured 24h-old biofilm. This observation is in accordance with the fact that fungal cells embedded in biofilms are less susceptible to conventional antifungal drugs as well as to antibodies (Fanning & Mitchell, 2012). Nevertheless, hyphae formation can be significantly impaired by the continuous presence of CAGTA that alter the surface of the hyphal cell wall. Other studies have reported comparable changes of *Candida* cell morphology when exposed to peptide P-113Du and P-113Tri (Lin et al., 2016) or marine polyunsaturated fatty acids (Thibane, Kock, Ells, Van Wyk, & Pohl, 2010). Our data are in line with initial evidence that other monoclonal and polyclonal antibodies, such as MAb 7D7 (Mishra, Ali, & Shukla, 2015) or anti-C3-RP Ab and MAb OKM1 (Bujdakova et al., 2008), also altered the development of *C. albicans* biofilm. Interestingly, recent data demonstrate that antibody-derived peptides, such as the Killer Peptide (KP), and molecules contained in commercial mouthwashes, are able to significantly impair *C. albicans* biofilm formation and maturation in terms of both biomass and cell metabolic activity (Paulone, Ardizzoni et al., 2017; Paulone, Malavasi et al., 2017). Taken together, these findings open new ways to alternative strategies to counteract fungal biofilm formation.

We can summarize that CAGTA are able to inhibit biofilm development depending on their concentration, and this inhibitory activity is focused on the initial phase of biofilm formation.



Antibodies confer protection against fungal infections by multiple mechanisms that include direct neutralization of fungi and their antigens, inhibition of the fungal growth, modification of gene expression, signaling and lipid metabolism, causing iron starvation, inhibition of polysaccharide release and biofilm formation (Lehrnbecher & Armstrong-James, 2017). In addition, they promote opsonization of fungi and phagocytosis, complement activation and antibody-dependent cell toxicity. (Elluru, Kaveri, & Bayry, 2015).

Previous studies observed the inhibitory activity of immunoglobulins during *Candida* growth, adhesion and biofilm formation. In this regard, IgG for *C. albicans* antigens inhibited the capacity of adherence of the yeast to polystyrene and fibronectin (Rodier et al., 2003); similar results were obtained using salivary secretory IgA (San Millán et al., 2000). In addition, Ding and colleagues observed that polyclonal sera obtained from a rabbit model of IC inhibited *C. albicans* growth and biofilm formation (Ding, Liu, Su, & Yan, 2014).

Nowadays, the development of new immunotherapy strategies against invasive candidiasis, which may improve the outcome of those patients with inappropriate host defense mechanisms, arouses an increasing interest. New immunotherapies, including vaccines, are driven by the progress made in the understanding of the molecular mechanisms underlying the pathogenesis of the infectious agent (Sampaio & Pais, 2017). Potential approaches include the administration of granulocytes, T cells, IFN- γ or therapy based on antibodies administration (Cassone, Bernardis, & Torosasantucci, 2005; Lehrnbecher & Armstrong-James, 2017; Magliani et al., 2005; Sampaio & Pais, 2017)

However, as a whole, this work warrant further studies to confirm the possible inhibitory activity of CAGTA, alone or combined with antifungal compounds. Moreover, identifying new antigens recognized by CAGTA can be the basis for the development of immunization protocols that might protect against *Candida* infections.



The following section refers to the work developed during my stay at Dr Naglik's laboratory of the King's College of London. The aim of the project was to increase our knowledge of *C. albicans* pathogenicity and the ability of specific *Candida* mycelial cell wall components to promote damage and generate an immunological response.

C. albicans is a pathogenic fungus with a wide capacity to adhere to and invade human buccal epithelial cells (Höfs, Mogavero, & Hube, 2016; Moyes et al., 2015; Naglik et al., 2011). The epithelial cells are the first barrier against pathogens and, regarding *C. albicans*, these cells are able to discriminate between yeast and hyphal form. In this way, hyphal morphotype can activate the MAPK pathway signaling response. This pathway includes the activation of the phosphorylation of MKP1, the transcription factor c-Fos and consensus DNA binding, which triggers and regulate the induction of cytokine responses (Moyes et al., 2016; Richardson et al., 2018). As previously explained, screening of a panel of mutant genes of *C. albicans* associated with the transition from blastospore to hyphae and pathogenicity, revealed that only *Ece1Δ/Δ* mutant was incapable of inducing this epithelial danger response, despite it formed normal hyphae and adhered to human buccal epithelial cells (Moyes et al., 2016). This gene was described by Birse and colleagues (Birse et al., 1993), and it is highly expressed during hyphae epithelial infection. Besides, Ece1p is a substrate for a kexine-like proteinase (Bader, Krauke, & Hube, 2008). Kex2 is a specific serine proteinase required for protein maturation (Yang et al., 2013). A murine model of systemic infection with *C. albicans kex2Δ/Δ* mutant showed attenuated virulence (Newport et al., 2003). In addition, the importance of Kex2p-mediated Ece1p processing was demonstrated using a *kex2Δ/Δ* null strain, which was unable to damage oral epithelia or induce p-MKP1/c-Fos mediated danger responses or cytokine secretion (Moyes et al., 2016). Ece1p presents seven consensus recognition points for Kex2p that generate eight different fragments of *C. albicans* Ece1p (Bader et al., 2008). Moyes and colleagues determined that the fragment Ece1-III_{62-93KR} produced damage and epithelial activation; it was named Candidalysin (Moyes et al., 2016).

The amphipathic properties of Ece1-III_{62-93KR} (SIIGIIMGILGNIPQVIQIIMSIVKAFKGNKR) coupled with the α-helical structure of



the N-terminal hydrophobic region indicated that this fungal peptide may act similarly to cationic antimicrobial peptides and peptide toxins such as melittin (honey bee) (Liu, Huang, Zhou, & Berne, 2005), magainin 22 (African clawed frog) (Bechinger & Salnikov, 2012) and alamethicin (*Trichoderma viride*) (Pieta, Mirza, & Lipkowski, 2012; Moyes et al., 2016).

Analysis of Ece1p amino acid sequences from different *Candida* species revealed the presence of additional putative Candidalysin toxins in *C. dubliniensis*, *C. tropicalis*, *C. albicans* 529L and *C. maltosa*. While the activity of the *C. albicans* Candidalysin has been characterized, there is no information regarding the activity of the Candidalysin analogs from other *Candida* spp. such as *C. tropicalis*, *C. dubliniensis* or *C. maltosa*. Even though the common features between the Candidalysins are likely to share common functions, the differences in the α -helix-forming regions and molecular rigidity may lead to variations in biological activity.

We know that *C. albicans* hyphal infection results in an up regulation of epithelia cytokines such as IL-1 α , IL-1 β , IL-6, IL-8, TNF- α , GM-CSF and others. This response regulates leukocyte trafficking and activates a strong antifungal response (Weindl et al., 2007). In our assay, we did not include TNF- α because it lacks reproducibility, and IL-8 due to that it is highly constitutive. On the contrary, the secretion of IL-1 α is widely regarded in association with damage induction (Suwara et al., 2014).

As we have mentioned, Ece1p is a substrate for Kex2p, a protease that cleaves proteins after lysine-arginine (KR) motifs, rendering fragments that terminate in KR (Bader et al., 2008; Moyes et al., 2016). Consequently, the Candidalysin was initially predicted to terminate in a dibasic lysine-arginine (KR) motif based upon Kex2p substrate specificity, and a peptide corresponding to this sequence (SIIGIIMGILGNIPQVIQIIMSIVKAFKGNKR) was capable of damaging epithelial cells and activating the c-Fos/MKP1 signaling circuits *in vitro*. However, subsequent analyses of hypha-secreted peptides revealed that the active secreted Candidalysin lacks the C-terminal arginine residue as a consequence of the carboxypeptidase Kex1p activity in a second processing step (Moyes et al., 2016). It is known that



following Kex2p cleavage, many proteins are further processed by Kex1p (Schmitt & Breinig, 2006).

To confirm that removal of the C-terminal arginine residue from immature Candidalysin was due to the function of Kex1p, we analyzed the c-Fos binding activity of a *kex1Δ/Δ* null mutant compared to its parental and matched revertant strains. c-Fos translocates to the nucleus and binds to its consensus DNA sequence site, which initiates the induction of downstream pro-inflammatory cytokines, including IL-1 α , IL-6, G-CSF, GM-CSF and IL-1 β (Moyes et al., 2010).

The dominant peptide secreted by the *kex1Δ/Δ* null mutant was immature Candidalysin (terminating in KR), whereas the *kex1Δ/Δ+KEX1* revertant showed WT-like secretion patterns, and recovered the c-Fos DNA binding activity of the wild type strain. *Kex1Δ/Δ* null mutant was unable to induce c-Fos, damage, p-MKP1 activation and cytokine profile secretion (Richardson et al., 2018), and therefore Kex1p activity appears as an important requirement for Candidalysin maturation being Candidalysin terminating in K residue the mature peptide. However, the biological reason for removal of the C-terminal arginine residue by Kex1p is unclear, as both immature (Ece1-III_{62-93KR}) and mature (Ece1-III_{62-93K}) Candidalysin are able to damage and activate epithelial cells, with Ece1-III_{62-93KR} being even more cytolytic than mature Candidalysin at lower concentrations (Moyes et al., 2016). Being an essential factor of the Candidalysin maturation (Richardson et al., 2018), we can suppose that this removal confers an evolutionary advantage to *C. albicans*, considering that Kex1p targets multiple proteins that are also required for fungal fitness and virulence (Richardson et al., 2018).

Moyes and colleagues postulated that the positively-charged C-terminal KR residues of Ece1-III_{62-93KR} might be critical for interacting with negatively-charged components of host membranes to promote lesion formation. In their work, the substitution of the KR motif to AA (alanine-alanine; Ece1-III_{62-93AA}) did not affect membrane intercalation, but significantly reduced the peptide's ability to permeabilize membranes, damage epithelial cells and induce calcium influx. In addition, Ece1-III_{62-93AA} still induced p-MKP1, c-Fos and the non-damage associated cytokine G-CSF but not the damage-associated cytokine IL-1 α , suggesting that Ece1-III_{62-93AA} can be recognized by epithelial immunity without damaging cells and



confirming that the positive C-terminus of Ece1-III_{62-93KR} is critical for lesion formation and damage induction in epithelial membranes (Moyes et al., 2016). Therefore, to determine whether the K motif ability to induce epithelial damage relied on its amino acid sequence or positive charge, we synthesized Candidalysins terminating in E (glutamic acid; negatively-charged) or H (histidine; positively-charged) and compared them to the wild-type Candidalysins (positively-charged K) and the Candidalysins terminating in A (neutral charge).

Concerning *C. albicans* Candidalysin wild type (K), as expected, we observed high damage induction, c-Fos/p-MPK1 activation and profile of pro-inflammatory cytokine secretion. These results were similar to those obtained by Moyes and colleagues, using Ece1-III_{62-93K} on TR146 epithelial cells (Moyes et al., 2016). Besides, the peptide presented the same damage pattern activity of the correspondent *C. albicans* strains SC5314 and BWP17+Clp30, after 2 hours of incubation with TR146 epithelial cell line (Moyes et al., 2010; Moyes et al., 2012; Moyes et al., 2016). Although it had been hypothesized that changing the charge of the C-terminal amino acid of Candidalysin would decrease its activity, in this work we observed the same pattern of damage induction and c-Fos/p-MPK1 activation by *C. albicans* Candidalysin terminating in E (negatively charged) and A (neutral charged). Intriguingly, Ece1-III₆₂₋₉₃ H (positive charge) did not promote damage induction of epithelial cell line, probably because of the conformation of the peptide and its final interaction with the epithelium. Our results did not agree with those of Moyes and colleagues who observed that Ece1-III₆₂₋₉₃ terminated in AA presented reduced damage activity on epithelial cells (Moyes et al., 2016).

Regarding the cytokines production, in agreement with the absence of damage induction (no LDH release), the peptide H induced less cytokine secretion, in comparison with the peptide K and the vehicle control. The lowest IL-1 β cytokine secretion reported at the highest K-peptide concentration can be related to the early damage registered in epithelial cells, which causes cell death and therefore, they cannot secrete cytokines anymore. Meanwhile, the constitutive cytokine IL-1 α was released immediately upon stimulation in a concentration dependent manner (Moyes et al., 2012). Besides, even low concentration of the peptides can induce cell damage and cytokines secretion (IL-1 α , IL-6, G-CSF, GM-CSF, IL-1 β). Similar results



were obtained by Moyes and colleagues using Ece1-III_{62-93K} on TR146 epithelial cells with the G-CSF and IL-1 α secreted cytokines (Moyes et al., 2016).

In this regard, based on the results obtained, C-terminal K residue seems not to be critical for interacting with negative charge of epithelial cell. Candidalysin terminating in neutral charge (A) presents the same damage pattern than the rest of *C. albicans* variant peptides. On the other hand, Candidalysin terminating with positive charge (H) does not generate damage and cytokine secretion, confirming that a C-terminal positive residue is not critical for interacting with the membrane.

On the other hand, Candidalysin variants of *C. dubliniensis* and *C. tropicalis* were able to induce a high release of LDH, indicating damage induction in spite of protein charge. In addition, all of them activated c-Fos and p-MKP1 which trigger and regulate proinflammatory cytokine responses.

Regarding the cytokine secretion, IL-1 α is constitutive and then immediately secreted into the medium upon damage stimulation of the epithelial cells promoted by the peptides K and H; these results are supported by Weindl and colleagues (Weindl, Wagener, & Schaller, 2011) and Moyes and colleagues (Moyes et al., 2010; Moyes et al., 2016; Moyes et al., 2011). The lowest IL-1 β , IL-6, GM-CSF and C-CSF cytokine secretion reported at the highest peptide concentrations can be related to the early damage registered in epithelial cells, which caused early cell death, and therefore they cannot secrete cytokines anymore.

Regarding *C. maltosa*, we do not have any previous information. Despite it is not considered a human pathogen, the effect of *C. maltosa* Candidalysin was very similar to those of *C. dubliniensis* and *C. tropicalis* in all the aspects studied and were related to the cell damage induction, c-Fos/p-MKP1 activation and cytokine secretion.

C. dubliniensis and *C. tropicalis* Candidalysin variants caused more damage on the epithelial cell line than that of *C. albicans*. While, wild type *C. dubliniensis* (CD36) and *C. tropicalis* (ATTC 750) strains induced cell damage, as evidenced by LDH release, they were unable to induce c-Fos and p-MKP1 activation, and correlated with a little increase of IL-1 α . Despite of that, *C. tropicalis* cells were unable to induce G-CSF, GM-CSF or IL-6, while *C. dubliniensis* hyphae were capable to promote c-Fos/p-MKP1 activation and a weak IL-6 cytokine secretion (Moyes et al., 2012).



Previous studies carried out by Dr Naglik's group revealed that Jpred secondary structure prediction software detected two different α -helix-forming regions in *C. albicans* (SIIGIIMGIL**GN**IPQVIQIIMSIVKAFKGMK) and *C. dubliniensis* (SIIGILTAI**LNNV**PQIINVITTIKSITGNK) Candidalysins separated by a spacer region, shown in bold. Intriguingly, *C. tropicalis* Candidalysin (ISFAGIVSSIINQLPSIIQIIGNIIKAGLVK) presented only a single α -helix-forming region without a spacer region. This feature suggests that *C. tropicalis* Candidalysin adopts a more rigid structure that would partially explain its great potency *in vitro*, compared with that of *C. albicans*. Differences among their peptide sequences may affect the interaction of the peptides with the epithelial cells, and cause damage to a variable extent. However, other consequences of this molecular rigidity are unknown and more studies are required to further characterize the signaling pathway and effector response of *C. dubliniensis*, *C. tropicalis* and *C. maltosa* Candidalysin analogues.

Analogue Candidalysin peptides of *C. tropicalis*, *C. dubliniensis* and *C. maltosa* exhibited greater potency *in vitro* than the Candidalysin of *C. albicans*. However, similarly to *C. albicans* Candidalysin, the C-terminal K residue of *C. tropicalis* and *C. dubliniensis* peptides seems not to be critical for interacting with the negative charge of epithelial cells.

C. albicans 529L is a clinical isolate unable to form hyphae, and in the presence of epithelial cells cannot activate MKP1, c-Fos, or cytokine production *in vitro*. However, it can colonize oral and vaginal mucosal surfaces of a murine model when compared to *C. albicans* SC5314 (Moyes et al., 2010; Rahman et al., 2007). Previous studies showed that hypha formation is essential for epithelial damage and cytokine induction in oral epithelial cells (Moyes et al., 2010). Even if it had been previously induced to form hyphae, once in contact with TR146 cells it became unable to maintain hyphal growth on epithelial cells resulting in low expression of *ECE1*, accompanied by c-Fos production but not c-Fos nuclear DNA binding, cytokine or damage of oral epithelial cells (Shirley, 2015).

In this study, we observed a paradoxical damage induction activity. Noticeably, LDH indicated the highest damage induction with the lowest peptide concentration. High peptide concentrations would activate a mechanism of defense of epithelial cells to



limit the effect of Candidalysin. The pro-inflammatory cytokine secretion profile exhibited the same pattern as LDH, with the exception of G-CSF. Regarding p-MKP1 and c-Fos expression in oral epithelial cells, it was evident only at the highest Candidalysin concentration assayed.

The results obtained with this variant Candidalysin peptide, might be explained by the different amino acid sequence of peptides from *C. albicans* 529L **SFLSIITALL**GNIPQ**IIQIIMGIV**KAFRGNK and *C. albicans* SC 5314 **SIIGIIMGIL**GNIPQ**VIQIIMSIV**KAFKGNK. Both sequences exhibit two distinct amyloidogenic regions, in red, which are predicted to form a α -helix separated by a spacer region. The different amino acid sequences observed in the hydrophobic region would partially explain the induction of damage registered for *C. albicans* 529L Candidalysin, considering that this region is critical for the epithelial activation and damage induction, as previously studied by Naglik's group.

In addition, Ece1-III has been seen to affect the mannoprotein structure (Shirley, 2015); however, the mannoprotein layer itself does not appear to contribute to the epithelial p-MKP1/c-Fos/cytokine/damage activation mechanisms. This observation may suggest that differences in the Ecep1-III sequences are critical for damage patterns in oral epithelial cell. This protein can bind or link together surface mannoproteins to provide structure or integrity, as postulated by Dr Naglik's research group, but further investigations are warranted to understand the role of the Candidalysin from *C. albicans* 529L during epithelial infection.

Generally, we observed that all Candidalysin mutants used in the assays presented a dosage effect on epithelia damage induction (LDH assay). These results suggest that despite being different in their amino acid sequences, the function of these peptides is conserved, and identified as a toxin related to the fungal pathogenicity

Here we provide a strong evidence on the effect of Candidalysin peptides from different *Candida* species on TR146 epithelial cells. These peptides are able to activate p-MKP1/c-Fos/cytokine/damage in oral epithelial cells *in vitro* and *in vivo*. However, it would be also interesting to determine whether they are able to induce c-Fos binding activity, considering that c-Fos translocates into the nucleus and binds

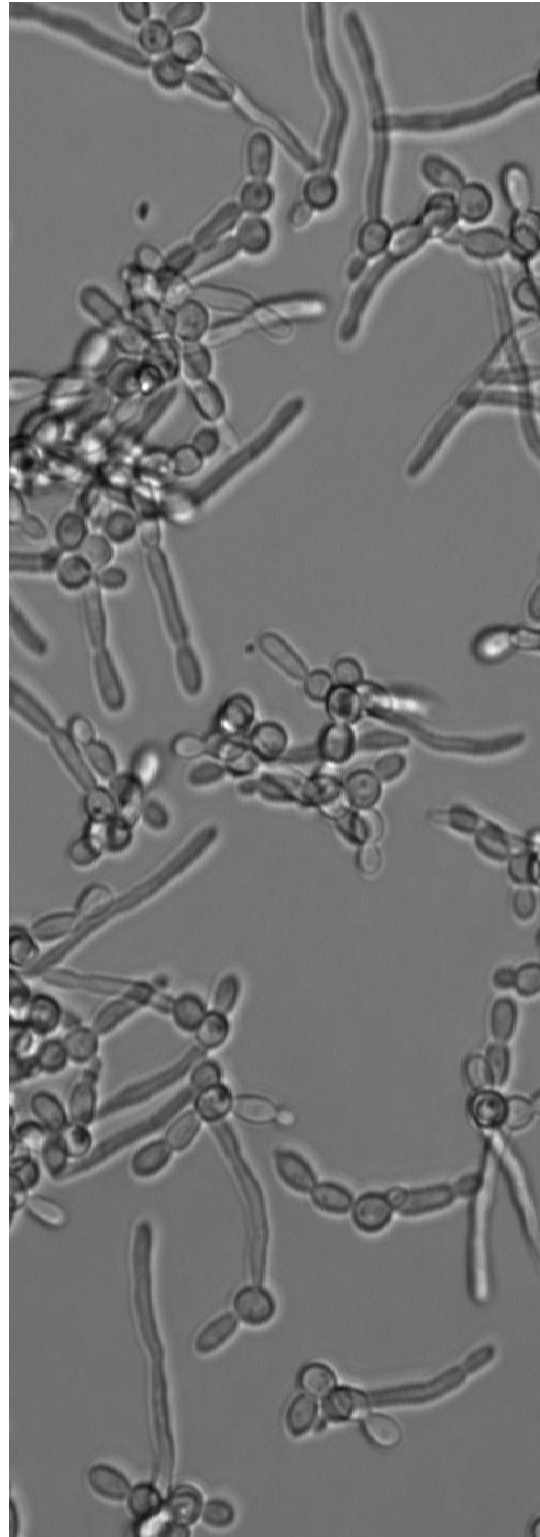


to its consensus DNA sequence site, which initiates the induction of downstream pro-inflammatory cytokines, including IL-1 α , IL-6, G-CSF, GM-CSF and IL-1 β (Moyes et al., 2010).

In addition, microbial interaction with mucosa surface is critical during an invasive process. Due to the complex interactions between *C. albicans* and epithelial cells, further studies are warranted to elucidate the Candidalysin functions considering that host immunological responses or hypha formation may not be the unique drivers of *C. albicans* pathogenity; fungal-bacteria interaction could play an important role during *Candida* infections and affect Ece1p expression.

The nature of Candidalysin sequence is also important in determining the ability of *C. albicans* to activate and damage oral epithelial cells and could provide novel targets for the development of new therapies to treat an infection caused by *Candida*. These features could be the basis for future investigations.

6 Conclusions





6

Conclusiones

Conclusions

1. En el modelo de infección por *C. albicans* en conejo, el título de anticuerpos anti-*Candida* de clase IgG aumenta tras el contacto repetido con el hongo en sucesivas inoculaciones.
 2. Los anticuerpos anti-Adh1 y anti-Met-6 podrían encontrarse entre los principales responsables de la mayor reacción de inmunofluorescencia frente a *C. albicans* de los sueros de los estadios tardíos en un modelo de candidiasis invasora en conejos; mientras que la respuesta más temprana de los anticuerpos anti-Eno-1, anti-Eno-pared y anti-Hwp1-N hace de sus correspondientes proteínas (Eno-1, Eno-pared y Hwp1-N) importantes inmunógenos durante las primeras fases de la infección
 3. La fracción de suero enriquecida en CAGTA de un modelo de CI de conejo incluye anticuerpos de distintas especificidades frente a antígenos cuya expresión está relacionada con la fase micelial de *C. albicans*. Además de la Hwp1 que ya está descrita, se incluyen Act1, Fas1, PGI1 y Phr1. Los sueros de pacientes con CI también reaccionaban con las proteínas expresadas por los clones
1. In the rabbit model of IC infected with *C. albicans*, anti-*Candida* IgG antibody titers increase after several repeated contacts with the fungus due to the successive inoculations.
 2. Anti-Adh1 and anti-Met-6 antibodies could be included among the most responsible for the immunofluorescence reaction against *C. albicans* from sera of the late phases of the infection in a rabbit model of IC, while the early response of anti-Eno-1, anti-CW-Eno and anti-Hwp1-N would convert their correspondent proteins (Eno-1, CW-Eno and Hwp1-N) important immunogens during the first stages of *Candida* infection.
 3. CAGTA-enr fraction of a rabbit model of IC contains antibodies with different specificities against antigens related to the mycelia phase of *C. albicans*, in addition to Hwp1, which had been previously described; we include Act1, Fas1, PGI1 and Phr1. Besides, it was confirmed that sera from patients with IC were able to recognize proteins expressed by positive clones previously selected

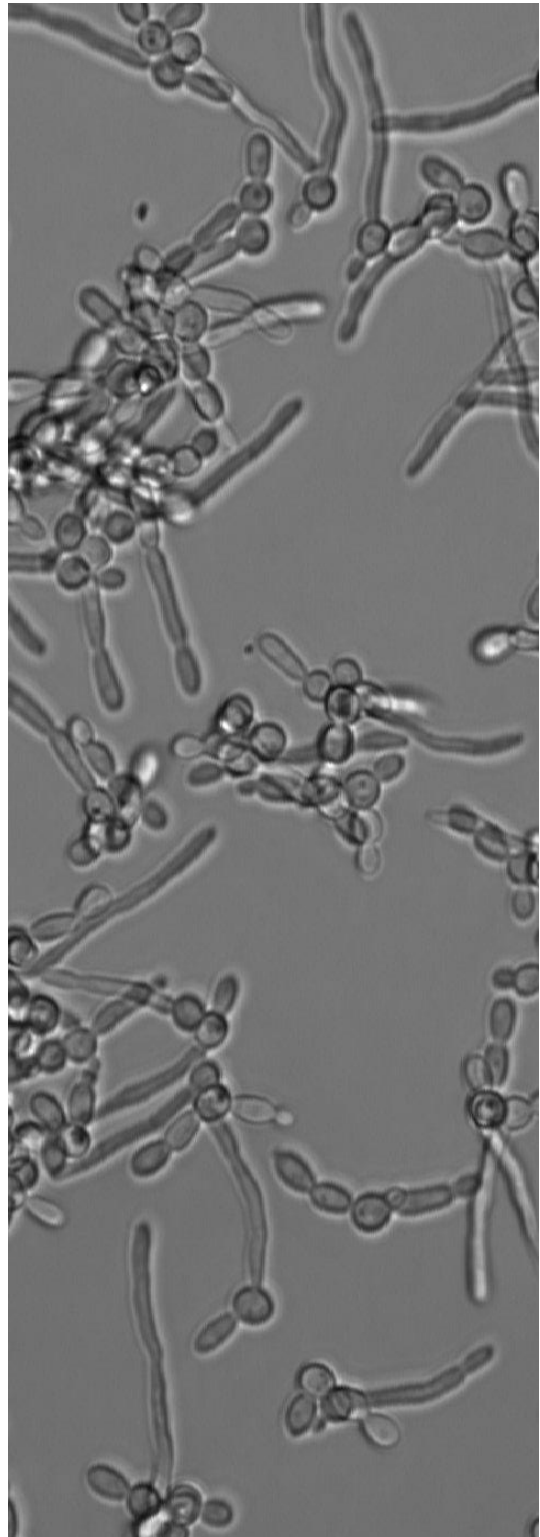


- previamente seleccionados con los CAGTA del modelo de candidiasis en conejo.
4. Las IgG de los sueros inmunes de conejos con CI reducen la actividad metabólica de las levaduras de *C. albicans in vitro*. Así mismo la principal actividad se observa en la fracción CAGTA-enr del suero del modelo de CI en conejo, afectando a la estructura de la pared de los tubos germinales.
 5. Los CAGTA purificados a partir de un modelo animal de CI en conejo muestran actividad fungicida frente a los tubos germinales de *C. albicans*.
 6. De un modo similar a lo registrado en el modelo animal, los sueros de pacientes con una CI causada por *C. albicans* reducen la actividad metabólica de las levaduras de *Candida in vitro*. Así mismo, la principal actividad se observa en la fracción CAGTA-enr de pacientes con candidemia, la cual afecta a la estructura de la pared de los tubos germinales. Los CAGTA purificados a partir de pacientes con CI tienen actividad fungicida.
 7. Los pacientes infectados por una especie de *Candida no-albicans* generan anticuerpos que muestran reacción cruzada con *C. albicans*, reduciendo su actividad metabólica y viabilidad, aunque con menor intensidad.
 8. Los CAGTA purificados inhiben la formación de biopelículas de *C. albicans* dependiendo de su concentración. La actividad inhibitoria de los CAGTA se centra en las fases iniciales de formación de la biopelícula.
 9. La actividad Kex1p es un requerimiento fundamental para la maduración del péptido Candidalysina, eliminando el aminoácido R del extremo C-terminal para generar el péptido maduro terminado en un residuo K.
- using CAGTA from a rabbit model of IC.
4. IgG from immune sera raised from a rabbit model of IC reduce the metabolic activity of *C. albicans* blastospore. In addition, the most important activity was observed in the CAGTA-enr fraction from that rabbit model of IC; this fraction is able to affect the wall of mycelia.
 5. CAGTA raised from a rabbit model of IC showed fungicidal activity against germ tubes of *C. albicans*.
 6. Similar to the results observed in the animal model, sera from patients with an IC caused by *C. albicans*, reduce the metabolic activity of *C. albicans in vitro*. In addition, the main activity was observed in the CAGTA-enr fraction from those patients, which affects the structure of the mycelia cell wall. Purified CAGTA from patients with IC revealed fungicidal activity.
 7. Patients infected with *Candida no-albicans* produce antibodies that showed cross-reactions with *C. albicans*, reducing its metabolic activity and viability, although with low intensity.
 8. CAGTA are able to inhibit *C. albicans* biofilm development depending on their concentration, and this inhibitory activity is focused on the initial phase of biofilm formation.
 9. Kex1p activity appears as an important requirement for Candidalysin maturation, the amino acid R of the C-terminal is eliminated in order to generate the mature peptide terminating in K residue.



10. La carga positiva del residuo K situado en el extremo C-terminal del péptido Candidalysina no parece ser el factor determinante de su interacción con las células epiteliales para causar daño celular y producir citoquinas.
 11. Los análogos de los péptidos de Candidalysina de *C. tropicalis*, *C. dubliniensis* y *C. maltosa* generan *in vitro* un patrón de daño a nivel celular en las células epiteliales mucho más elevado que la Candidalysina de *C. albicans*. Los residuos C-terminales de los péptidos de *C. tropicalis* y *C. dubliniensis* no parecen ser un factor clave en la interacción con la carga negativa de las células epiteliales.
 12. Todas las variantes de Candidalysina produjeron un efecto dañino dosis-dependiente sobre las células epiteliales (ensayos LDH). Estos resultados sugieren que, a pesar de diferir entre ellas en sus secuencias de aminoácidos, la función de estos péptidos se conserva y se identifica como una toxina relacionada con la patogenicidad del hongo.
10. The positive charge of C-terminal K residue of Candidalysin does not seem to be critical for interacting with epithelial cells to generate cell damage and cytokine secretion.
 11. Analogue Candidalysin peptides of *C. tropicalis*, *C. dubliniensis* and *C. maltosa* exhibited greater potency *in vitro* than the Candidalysin of *C. albicans*. However, similarly to *C. albicans* Candidalysin, the C-terminal K residue of *C. tropicalis* and *C. dubliniensis* peptides seems not to be critical for interacting with the negatively charged surface of epithelial cells.
 12. All Candidalysin variants presented a dosage effect on epithelia damage induction (LDH assays). These results suggest that despite being different in their amino acid sequences, the function of these peptides is conserved, and identified as a toxin related to the fungal pathogenicity.

5 References





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