RESEARCH ARTICLE

Carbon metabolism snapshot by ddPCR during the early step of *Candida albicans* phagocytosis by macrophages

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ABSTRACT

During *Candida* macrophage interactions, phagocytosed yeast cells feed in order to grow, develop hyphae and escape. Through numerous proteomic and transcriptomic studies, two metabolic phases have been described. A shift to a starvation mode is generally identified as early as one-hour post phagocytosis, followed by a glycolytic growth mode after *C. albicans* escaped from the macrophage. Healthy macrophages contain low amounts of glucose. To determine if this carbon source was sensed and metabolized by the pathogen, we explored the transcription level of a delimited set of key genes expressed in *C. albicans* cells during phagocytosis by macrophages, at an early stage of the interaction. This analysis was performed using a technical digital droplet PCR approach to quantify reliably the expression of carbon metabolic genes after 30 min of phagocytosis. Our data confirm the technique of digital droplet PCR for the detection of *C. albicans* transcripts using cells recovered after a short period of phagocytosis. At this stage, carbon metabolism is clearly oriented towards the use of alternative sources. However, the activation of high-affinity glucose transport system suggests that the low amount of glucose initially present in the macrophages is detected by the pathogen.

Keywords: *Candida albicans*; ddPCR; macrophages; carbon metabolism; glucose transport; glucose sensing and lipid β-oxidation

INTRODUCTION

The polymorphic yeast *C. albicans* is both a powerful commensal and a pathogen to humans. This pathogenic yeast can colonize a wide range of organs and bodysites (Odds 1988) and is the cause of infections, especially in immunocompromised patients. During systemic infections, macrophages are key phagocytes that migrate to the site of infection and mediate killing of the pathogen as part of the primary line of defense against microbial infections (Erwig and Gow 2016). Most defense against systemic *Candida* infection relies on the capacity of phagocytes to ingest and destroy fungal cells. Macrophage migrates towards the fungus by chemotaxis, resulting in the interaction of the fungal cell surface pathogen-associated molecular patterns with macrophage recognition receptors, which leads to phagocytosis

into a phagosomal compartment (Cannon and Swanson 1992; Romani 2011). Subsequently a series of lysosomal vesicle fusion events leads to the formation of the toxic phagolysosome. However, *C. albicans* has evolved mechanisms that promote its survival and replication inside this particular host cell. Among its virulence determinants, survival at 37° C, pH and osmolarity adaptation, secretion of lytic enzymes, alteration of the immune response, morphological changes such as a transition between yeast and hyphae occur during macrophage infection (Noble, Gianetti and Witchley 2017). Hyphal filaments are formed at the same time as the fungus escapes from the phagocyte, creating an inflammasome-dependent lytic death by pyroptose (Uwamahoro *et al.* 2014; O'Meara and Cowen 2018). However, this is not a macrophage killing dominant mechanism. Tucey *et al.* (2018) proposed that in *C. albicans* activated macrophages, a shift to Warburg metabolism dependent on glucose for survival settles in. By depleting glucose level at the infection site, *C. albicans* also triggers massive death of macrophages.

Description of the interaction between *C. albicans* and macrophages by transcriptomic and proteomic approaches using *in vitro* models, reveal unambiguously a dramatic reprogramming of carbon metabolism gene transcription towards a starvation mode (Lorenz and Fink 2002; Lorenz, Bender and Fink 2004; Ferna´ndez-Arenas *et al.* 2007; Jime´nez-Lo´pez and Lorenz 2013; Kitahara *et al.* 2015; Munoz *et al.* 2019). Fungal response to macrophage phagocytosis occurs in two successive steps. First, between 1 and 4 h of phagocytosis, alternative carbon metabolism including β-oxidation, glyoxylate and gluconeogenesis pathways are activated. All these data suggest that glucose metabolism is not essential to *C. albicans* survival in macrophages. Once escaped from macrophages, induction of glycolysis initiates, to be prominent at step 6–9 h post infection to sustain extracellular replication (Lorenz, Bender and Fink 2004; Jime´nez-Lo´pez and Lorenz 2013; Tucey *et al.* 2018).

However, in a previous study, we have shown that hexokinase and hexokinase/glucokinase single and double deletion mutants (*Cahxk2*Δ/Δ, and *Cahxk2glk1*Δ/Δ) were both significantly hypovirulent in a macrophage model (Laurian *et al.* 2019). Moreover, other evidences could suggest that glucose use by the pathogen may occur during the first steps of its interaction with macrophages (Segin and Le Pape 1994; Prigneau *et al.* 2003; Luongo, Porta and Maresca 2005). Nuclear magnetic resonance studies of non-infected and primed macrophages reveled an intracellular glucose content reaching 5 mM (Segin and Le Pape 1994) which corresponds to the concentration level of the bloodstream (Barelle *et al.* 2006; Brown *et al.* 2014). Depending on the cellular compartment where this hexose is stored and how fast it is used by the macrophage itself, glucose could then be available in the vacuole or in the lumen of the phagolysosome during the very early steps of phagocytosis. Moreover, the expression of *CaGS*, an hexose transporter later renamed *CaHGT12*, is induced during macrophage infection (Luongo, Porta and Maresca 2005). *In vitro,* this gene is expressed in the presence of low glucose concentrations. Similarly, the *CG13* gene encoding a putative glucose sensor Snf3-like, is specifically expressed during macrophage infection (Prigneau *et al.* 2003).

To investigate the carbon metabolic profile at the initial step of phagocytosis, a delimited transcriptomic study was undertaken using only phagocytosed yeasts collected 30 min post infection. Expression analysis of targeted genes, implicated in central carbon metabolism and positioned at key points was conducted. To carry out this study, we have tested the innovative, sensitive and reliable technique of digital droplet PCR (ddPCR) to detect *C. albicans* transcripts at an early stage of phagocytosis.

MATERIALS AND METHODS

Strain and growth conditions

Candida albicans wild type strain (*SC5314*) was routinely cultivated in liquid YPGly medium (1% yeast extract, 1% peptone, 2% glycerol) at 30°C under agitation (180 rpm). The murine macrophage cell line J774A (ATCC TIB-67) was maintained in DMEM Glutamax (Fisher) supplemented with 10% decomplemented FBS (fetal bovine serum, Gibco) and 50 µg/mL of penicillin and streptomycin (Gibco). Before the infection experiments, macrophages were cultured in complete RPMI (cRPMI) medium (RPMI-1640, R8755, Sigma), with L-glutamine, without phenol red and supplemented with 10% decomplemented FBS, 1 mM sodium pyruvate, 2 g/L sodium bicarbonate and 50 µg/mL of penicillin and streptomycin (Gibco) at 37°C in 5% CO_2.

Infection of Macrophages J774 by *C. albicans*

The day before the infection experiment, *C. albicans* cells were grown overnight in YPGly medium containing 2% glycerol at 30° C under agitation (180 rpm). 3 × 10^7 macrophages were seeded overnight in 75 cm^2 flasks (Falcon) containing cRPMI medium at 37°C in 5% CO_2. The Multiplicity Of Infection (MOI) of 1M:3Y was selected after testing the MOI 1M:2Y because it allowed to recover a higher quantity of RNA, suitable to pursue with ddPCR experiments. On the day of the infection experiment, 9 × 10^7 stationary phase yeast cells were transferred into serum-free RPMI medium (named sfRPMI 0.2% glycerol). This medium is composed of RPMI 1640 (R1383, Sigma) with L-Glutamine, without glucose, sodium bicarbonate and phenol red supplemented with 1 mM sodium pyruvate, 2 g/L sodium bicarbonate, 50 µg/mL of penicillin and streptomycin (Gibco) and 0.2% glycerol. This medium was not supplemented with FBS, which is known as a strong inducer of filamentation. This led us to avoid the transcriptomic changes linked to hyphae formation before yeast cells were internalized in macrophages, in order to assess the yeast to hyphae transition specifically induced inside macrophages. The cRPMI medium was removed from the adherent macrophages, and phagocytes were infected with the prepared yeast cells for 30 min at 37° C in 5% CO_2 at a MOI of 1M:3Y. As a control, 9 × 10^7 yeast cells were cultured in the absence of macrophages in the same medium (sfRPMI 0.2% glycerol). This led us to assess the transcriptional changes that occurs specifically upon internalization inside macrophages.

Extraction of phagocytosed yeasts

After 30 min of co-incubation, plates were kept on ice to stop phagocytosis. Supernatants were removed and kept on ice. Unbound yeast cells were removed from adherent infected macrophages by washing with PBS, and pooled with free-yeast in supernatants before quantification using KOVA Slide. The internalization rate was assessed as follows: (initial inoculum-unbound cells)/initial inoculum x 100. Macrophages were scrapped twice in 10 mL of cold water, collected by centrifugation (5 min at 4500 *g*) and resuspended in macrophage lysis buffer (0.2% TritonX100, 50 mM Tris-HCl pH7.5, 2 mM EDTA). Macrophages were lysed by succession of six rounds of 30 sec grindings using Vortex Genie followed by 5 min on ice. The complete lysis of phagocytes was checked by microscopy. Yeasts were collected by centrifugation for 10 min at 4500 *g* at 4°C, washed in cold water and resuspended in 1 mL of Tri Reagent (Euromedex) for RNA extraction.

Analysis of *C. albicans* morphology

Cells under single yeast form and cells starting pseudo-hyphae formation were manually quantified from phase contrast images taken with an inverted microscope (Evos Cell Imaging). A total of 1500 cells corresponding to four randomly selected fields were counted using the Adobe Photoshop Counting tool.

RNA extraction and cDNA generation

Total RNA was extracted either from *C. albicans* cells grown in liquid cultures, either from yeasts internalized and recovered from macrophages. To prepare total RNA extracts, yeast cells alone and yeast cells extracted from phagocytes after internalization were suspended in 1 mL of Tri Reagent (Euromedex) and then broken in the presence of 450 µL of acid-washed glass beads, using a PreCellys 24 (Bertin) device (two bursts of 6500 rpm for 40 sec). After centrifugation of the lysate at 12 000 *g* for 15 min at 4° C, the supernatant was washed with 200 µL of chloroform and centrifuged at 12 000 *g* for 15 min at 4° C. RNA was precipitated by adding one volume of isopropanol and an incubation step at −20° C for 20 min. Total RNA were collected by centrifugation (12 000 *g* for 15 min at 4° C) and washed with 1 mL of 70% ethanol. The pellet was air-dried and suspended in 50 µL of DEPC (Diethylpyrocarbonate, Sigma) treated water.

Quantitative PCR (RT-qPCR) experiments

For reverse transcription-quantitative PCR (RT-qPCR) experiments, 10 μg of total RNA extract were treated with DNase I (Ambion). Then, ReVertAid H Minus reverse transcriptase (Thermo Scientific), was used as described by the manufacturer, to generate cDNAs. For each target analyzed by ddPCR, the specificity of the primers was tested by RT-qPCR. RT-qPCR experiments were performed with the CFX 96 Bio-Rad light cycler using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad). Relative quantification was based on the 2ΔCT method using CaACT1 (actin) as calibrator. The amplification reaction conditions were as follows: 95°C for 1 min, 40 cycles of 95° C for 15 sec, 60° C for 30 sec and the final step 95°C for 10 sec. A melting curve was generated at 95° for 10 sec, 65°C for 5 sec with an increment of 0.5°C until 95°C at the end of each PCR cycle, to check that a specific product was amplified. Primers used in this study are presented in Supplementary data Table 1.

Transcripts quantification by ddPCR

The ddPCR was performed using a QX100 Droplet Digital PCR System (Bio-Rad, California, USA) and two negative controls (non-retrotranscribed ARN and molecular grade water) were included for each gene analyzed. The optimized ddPCR reaction mix conditions retained were a final reaction volume of 22 μL containing: 2 × QX200 ddPCR EvaGreen Supermix (Bio-Rad), 900 nM forward and reverse primers and 5.5 μL of 50 ng/µL cDNA sample. Droplets were generated with the QX100 Droplet Generator (Bio-Rad) in a cartridge containing 20 μL of the reaction mix and 60 μL of Droplet Generation Oil for EvaGreen (Bio-Rad). The entire emulsion volume was transferred from the cartridge to a 96-well PCR plate (Bio-Rad) and the PCRs were performed on a T100 Thermal Cycler (Bio-Rad). Optimal thermocycling conditions retained were: DNA polymerase activation of 95°C for 5 min, then 35 cycles of two steps of 95°C for 30 sec for denaturation and 60°C for 60 sec for hybridization and elongation, followed by two steps of 4°C for 5 min and 90°C for 5 min for droplet stabilization. According to Bio-Rad recommendations, a temperature ramp of 2°C/sec was fixed on all PCR steps and the lead was heated at 105°C. Droplets were then analyzed for fluorescent amplitude using the QX100 Droplet Reader (BioRad). Samples showing more than 10 000 droplets were analyzed using the QuantaSoft Analysis Pro software version 1.0.596 (BioRad), and thresholds were set manually. Placement of the threshold was set for each assay by visually inspecting the amplitude plot for a negative control sample and placing the threshold between the cluster of positive droplets and the cluster of negative droplets.

Two reactions based on two different biological duplicates (repeated twice) of phagocytosed yeasts and yeast grown in the presence of glycerol were run. Data are expressed as a number of amplified copies per 20 µL well. For each gene, the indicated value corresponds to the ratio of the number of amplified copies detected in phagocytosed yeast in serum-free RPMI 0.2% glycerol to the number of copies detected after growth in sfRPMI 0.2% glycerol.

RESULTS

Design of the experimental approach and choice of targets

For phagocytic experiments, yeasts are usually maintained on YP medium containing 2% glucose (Prigneau *et al.* 2003; Lorenz, Bender and Fink 2004; Ferna´ndez-Arenas *et al.* 2007; Dementhon, El-Kirat-Chatel and Noe¨ l 2012; Kitahara *et al.* 2015). Then, infections of phagocytes with yeast are also performed routinely in the presence of RPMI medium containing 0.2% glucose. To be able to highlight a potential impact of the absence or presence of glucose on the transcription of phagocytosed yeast genes, glucose was not added to the yeast preculture and to the medium used for our phagocytic experiments. Likewise, serum, a strong inducer of germ tube formation, most likely due to the presence of glucose (Hudson *et al.* 2004), has not been incorporated either to the RPMI medium used for phagocytosis. Glycerol was used as carbon source instead of glucose. Yeast cells from a *C. albicans* preculture (YP 2% glycerol) were transferred either to sfRPMI 0.2% glycerol or to sfRPMI 0.2% glycerol containing macrophages at a MOI of 1:3. *C. albicans* cells, observed when cultured alone in sfRPMI 0.2% glycerol (Fig. 1A), revealed a majority of cells under the single yeast morphology (97%±1%) (Fig. 1C). This observation showed that hyphae formation was not induced when glycerol was used as a carbon source in the absence of serum. After 30 min of coincubation with macrophages (Fig. 1B), only 40% of the yeasts were internalized (Fig. 1D). The non-ingested yeasts were removed before internalized yeasts were released for RNA extraction.

Then, fungal transcripts were detected by a ddPCR approach. Indeed, this technique has proven itself to detect *in vivo* and *in vitro* microbial contaminations (Deshmukh *et al.* 2016; Talarico *et al.* 2016; Montenegro *et al.* 2019). The strength of this technique lies in the evidence that the reaction mix is distributed into separate droplets. After thermal cycling, count of the copy number of cDNA is realized by individual screening by fluorescence measurement without standard (Bhat *et al.* 2009). This technique allows a direct visualization of the amplified products at the single droplet level. It seemed particularly well suited to us given the low percentage of internalized cells.

Table 1. Key metabolic genes targeted in this study and function of their gene products.

Gene	*GenBank* number	Fonction
CaHGT3	XM_710 040.2	Putative glucose transporter, major facilitator superfamily
CaHGT4	XM_718 080.1	Glucose and galactose sensor
CaHGT9	XM_01 947 5543.1	Putative glucose transporter, major facilitator superfamily
CaHXT10	XM_01 947 5528.1	Putative sugar transporter
CaHGT12	XM_715 292.1	Glucose, fructose, mannose transporter, major facilitator superfamily
CaHGT17	XM_717 658.2	Putative glucose transporter, major facilitator superfamily
CaHGT19	XM_712 726.1	Putative glucose and myo/inositol transporter, major facilitator superfamily
CaHXK2	XM_712 312.1	Hexose phosphorylation, glycolysis
CaGLK1	XM_705 084.2	Glucose and mannose phosphorylation, glycolysis
CaPFK1	XM_716 783.1	Phosphofructokinase alpha sub-unit, glycolysis
CaPDC12	XM_01 947 5363.1	Putative pyruvate decarboxylase, fermentation pathway
CaPDX1	XM_717 131.1	Pyruvate dehydrogenase, essential component of the mitochondrial pyruvate dehydrogenase complex , links glycolysis to the Krebs cycle
CaCTF1	XM_717 453.2	Putative zinc-finger transcription factor, activation of genes required for fatty acid degradation
CaFAT1	XM_706 233.2	Long-chain fatty-acid transporter
CaPXA1	XM_713 564.2	Putative peroxisomal fatty acids transporter, ABC family transporter
CaPXA2	XM_711 231.2	Putative peroxisomal fatty acids transporter, ABC family transporter
CaACB1	XM_01 947 5477.1	Acyl-CoA transporter
CaFAA2–1	XM_709 191.2	Predicted long chain fatty acid-CoA ligase
CaFAA2–2	XM_714 877.2	Predicted acyl-CoA synthetase
CaFAA2–3	XM_710 112.1	Predicted acyl CoA synthetase
CaFAA4	XM_714 261.1	Acyl-CoA synthase involved in long-chain fatty acid uptake
CaFOX3	XM_716 682.1	Putative peroxisomal 3-oxoacyl-CoA thiolase
CaSFC1	XM_716 748.2	Putative succinate-fumarate transporter, transports cytoplasmic succinate into the mitochondrial matrix in exchange of fumarate
CaICL1	XM_707 139.1	Isocitrate lyase, glyoxylate cycle
CaPCK1	XM_713 578.1	Phosphoenolpyruvate carboxykinase, gluconeogenesis
CaALS3	XM_705 343.2	Cell wall adhesin
CaHWP1	XM_704 869.2	Hyphal cell wall protein

To address the question of *C. albicans* carbon metabolism during the very early step of phagocytosis, we have analyzed the expression of key genes that could allow to draw a brief metabolic portrait (Table 1). Genes encoding signaling pathway elements (receptors, transcription factors), transporters and proteins involved in catalytic functions, were selected.

To check the activation of glycolytic gene transcription, targets implicated in glucose detection, transport and metabolism in *C. albicans* were considered. *C. albicans* possesses a large family of predicted hexose transporters and sensor (Fan, Chaturvedi and Shen 2002; Brown, Sexton and Johnston 2006). The impact of glucose on the transcription of these transporters has been partially described and some of them have been characterized (Fan, Chaturvedi and Shen 2002; Brown, Sexton and Johnston 2006). Consequently, *CaHGT4,* a high-affinity sugar sensor (Brown, Sexton and Johnston 2006), *CaHGT3, CaHGT19, CaHGT9,* low affinity hexose transporters and *CaHGT12, CaHGT10* and *CaHGT17,* high-affinity hexose transporters (Fan, Chaturvedi and Shen 2002; Luo, Tong and Farley 2007) have been chosen (Fig. 5). Analysis of their expression level could provide information on the amount of glucose detected by *C. albicans* during the early step of phagocytosis. In *C. albicans*, glucose phosphorylation is mainly performed by the hexokinase *CaHXK2* and to a lesser extent by *CaGLK1,* a glucokinase (Laurian *et al.* 2019). Transcription of *CaHXK2* and *CaGLK1* genes implicated in the first step of glycolysis was then considered. To set a second marker of the glycolytic pathway, *CaPFK1*, a phosphofructokinase which catalysis the third step of the glycolysis that cannot be reversed and has been well characterized in *C. albicans* (Lorberg *et al.*1999), was also listed. Then, at subsequent stages, we considered *CaPDC12*, pyruvate decarboxylase and *CaPDX1*, pyruvate dehydrogenase, that direct the metabolic flow towards fermentation or respiration, respectively (Fig. 5).

A shift in gene expression towards alternative carbon utilization through the up regulation of *C. albicans* pathways such as gluconeogenesis, glyoxylate cycle and β-oxidation of fatty acids is observed during transcriptomic and proteomic studies of the interaction with macrophage (Lorenz and Fink 2001; Barelle *et al.* 2006; Piekarska *et al.* 2008 Jime´nez-Lo´pez and Lorenz 2013, Kitahara *et al.* 2015; Munoz *et al.* 2019). In *C. albicans*, phagocytosis upregulates CaIcl1, the principal enzyme of the glyoxylate cycle, a metabolic pathway that permits the use of two-carbon compounds as carbon source and is the main precursor of gluconeogenesis. *CaPCK1* encodes the gluconeogenic enzyme responsible for phosphoenolpyruvate synthesis and is a strong actor of metabolic flexibility in *C. albicans* (Barelle *et al.* 2006). A lot of data confirms the induction of *CaICL1* and *CaPCK1* in macrophages (Lorenz and Fink 2001; Barelle *et al.* 2006; Jime´nez-Lo´pez and Lorenz 2013). The analysis of their expression level will be used as marker (Fig. 5).

In a glucose-poor environment, *C. albicans* might produce glucose from fatty acids by using β-oxidation, glyoxylate cycle and gluconeogenesis pathways. Thus, the expression of genes involved in lipid metabolism and β-oxidation of fatty acids was also monitored. To be used, fatty acids must be imported across membranes by permeases, then activated by acetyl CoA synthetases, before integrating lipid catabolism pathways. To evaluate the expression of genes implicated in these different steps,

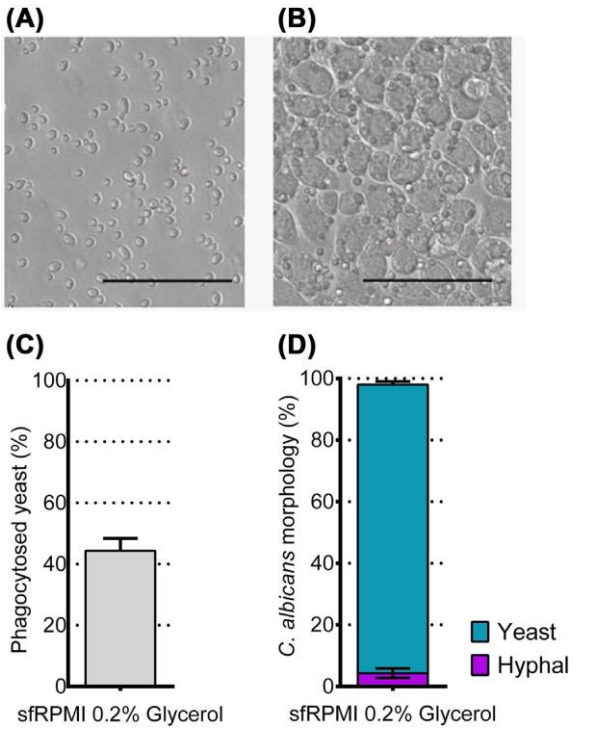

Figure 1. Representative picture of stationary phase *C. albicans* cells grown in the presence of sfRPMI glycerol 0.2% (A) and after 30 min of cocultivation with J774 macrophages in culture flasks at MOI 1:3 in sfRPMI 0.2% glycerol (B). The scale bar represents 50 μm. Percentage of phagocyted yeasts (C) and yeasts with hyphal development (D).

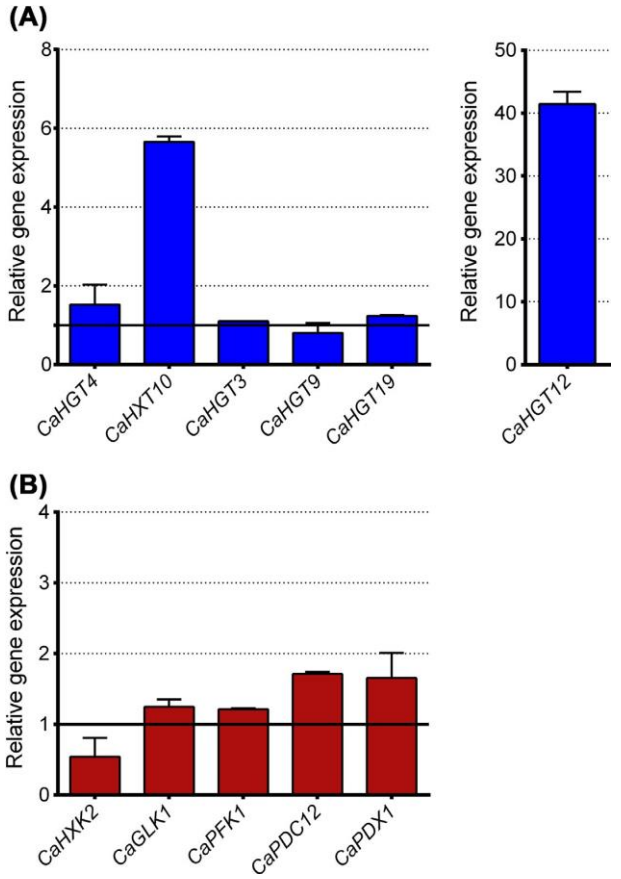

Figure 2. Glucose sensing, transport and metabolism gene expression. (A) Glucose sensor and transporter genes. Due to its high level of expression, *CaHGT12* was positioned on a separate diagram. (B) Genes implicated in the different stages of glycolysis. For each gene, the value corresponds to the ratio of the number of amplified copies detected in phagocytosed yeasts to the number of copies detected after growth in in sfRPMI 0.2% glycerol (black line). Results represent a mean of two gene expression values obtained with two biological replicates, except for *CaHGT3* for which only one value vas available.

CaPXA1, *CaPXA2*, *CaACB1*, *CaFAT1* (lipid transporters), *CaFAA 2–1*, *CaFAA 2–2*, *CaFAA 2–3*, *CaFAA4* (acetyl CoA synthetases), *CaFOX3*, (β-oxidation) were considered. In addition, the expression of *CaSFC1*, a mitochondrial succinate fumarate transporter, whose function could be to link β-oxidation of fatty acids and the glyoxylic cycle (in peroxysomes) to Krebs cycle (in mitochondria) and *CaCTF1*, a transcription factor necessary for growth on fatty acids that regulates the expression of several genes encoding enzymes of β-oxidation (Ramirez and Lorenz 2009) were analyzed (Fig. 5).

Our list of targets was completed by the analysis of two hyphae-specific genes: *CaALS3* and *CaHWP1*. *CaHWP1* is encoding a hyphal wall protein and *CaALS3* an adhesin agglutinin-like protein (Biswas, Van Dijck and Datta 2007; Almeida *et al.* 2008). The detection of transcripts of these genes could give information on the proper progress of intracellular yeast to hyphae development, under our experimental conditions (Fig. 5).

State of the *C. albicans* carbon metabolic profile after 30 min of phagocytosis by macrophages

To answer our initial question: is there any glucose use during the first step of macrophage phagocytosis? First, we analyzed the expression profile of genes implicated in glucose detection, transport and metabolism, in yeasts that have been internalized or not by macrophages (Fig. 2). While the expression level of the low-affinity glucose permeases *CaHGT3*, *CaHGT9* and *CaGHT19* remained equivalent in both conditions, transcription of genes encoding *CaHGT12*, *CaHXT10*, *CaHGT17*, high-affinity transporters dedicated to traces of glucose and the high-affinity glucose sensor *CaHGT4* was significantly activated following phagocytosis (Fig. 2). *CaHGT12* overexpression (X40) was particularly striking and suggests a special role for this hexose transporter. Those data show that expression of genes being part of the high-affinity glucose transport and sensing system in *C. albicans* is not repressed inside the macrophage and suggest that a low amount of glucose might be present.

By contrast, quantification of the hexokinase gene transcription revealed that *CaHXK2* was significantly under expressed. Surprisingly, this was not the case for the glucokinase gene *CaGLK1* which was slightly more expressed in the macrophages than when cultivated in the presence of glycerol (Fig. 2). This this could be explained by the different modes of regulation of these two genes (Laurian *et al.* 2019) and highlights that glucokinase and hexokinase, although both enzymes phosphorylate glucose and fructose, may not have totally redundant functions. In *C. albicans*, hexokinase and glucokinase transcription is induced in the presence of low (0.1%) and high (2%) amounts of glucose (Laurian *et al.* 2019). The transcription level of hexose kinase genes could also suggest that if glucose is present, it is in trace amounts. Transcription level of *CaPFK1*, another glycolytic gene (phosphofructokinase catalyzes the third step of glycolysis) was similar to that of *CaGLK1*. This confirms that apart from hexokinase, glycolytic genes are not repressed during the first step

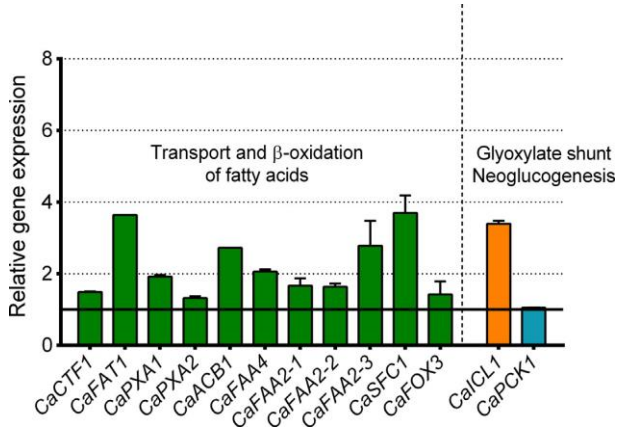

Figure 3. Expression of alternative carbon metabolism target genes. For each gene, the value corresponds to the ratio of the number of amplified copies detected in phagocytosed yeasts to the number of copies detected after growth in in sfRPMI 0.2% glycerol (black line). Results represent a mean of two gene expression values obtained with two biological replicates, except for *CaFAT1, CaACB1, CaPCK1,* for which only one value was available.

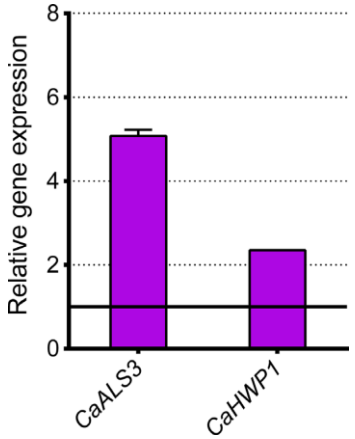

Figure 4. Expression of hyphae-specific target genes. For each gene, the value corresponds to the ratio of the number of amplified copies detected in phagocytosed yeasts to the number of copies detected after growth in in sfRPMI 0.2% glycerol (black line). Results represent a mean of two gene expression values obtained with two biological replicates, except for *CaHWP1* for which only one value vas available.

of phagocytosis but only weakly expressed. The analysis of the expression of *CaPDC12* and *CaPDX1,* pyruvate decarboxylase and pyruvate dehydrogenase, respectively, was intended to show whether the metabolism of pyruvate was oriented towards respiration or fermentation. Both genes were similarly expressed (X1.65). This could reflect disparity of the transcript level within the yeast population. Yeast cells may not have been internalized simultaneously and consequently exposed during the same time to the anoxic environment of the macrophage cell.

Studies conducted on *C. albicans* metabolism during phagocytosis by macrophages from one to 4 h of infection revealed the utilization of alternative carbon sources rather than glycolysis (Lorenz and Fink 2002; Diez-Orejas and Ferna´ndez-Arenas 2008; Tucey *et al.* 2018). The transcription of genes involved in transport and *β*-oxidation of fatty acids was then analyzed (Fig. 3). To be metabolized, fatty acids must undergo transmembrane transport through the plasma membrane (*CaFAT1*) and be addressed to the peroxisomes (*CaPXA1, CaPXA2s).* Prior metabolization by the *β*-oxidation pathway (CaFOX3), fatty acids must be activated by fatty acyl-CoA synthetases (*CaFAA2–1, CaFAA2–2, CcFAA2–3, CaFAA4, CaACB1*) and transcription of the genes implicated must be activated by transcription factors (*CaCTF1*). Globally, all of them were significantly overexpressed after 30 min of phagocytosis, suggesting that this alternative pathway is important to assess *C. albicans* metabolism under those conditions (Fig. 3). The mitochondrial succinate fumarate shuttle gene *CaSFC1* that links fatty acids catabolism and gluconeogenesis was also overexpressed.

Finally, we analyzed the transcription of two key genes of the glyoxylic cycle (*CaICL1*) and gluconeogenesis pathway (*CaPCK1*). Our data show a strong activation of the transcription of *CaICL1* (x.3.2), which suggests that the glyoxylic cycle may actively contribute to *C. albicans* metabolism at this early stage of phagocytosis (Fig. 3). By contrast, the transition from a glycerol medium to the macrophage does not seem to induce an overexpression of *CaPCK1,* a gluconeogenesis reporter gene.

To ensure that the activated metabolic program after 30 min of phagocytosis is part of a more general virulence process which includes destruction of macrophage as the same time as *C. albicans* produces hyphae and escapes, we analyzed the expression of two hyphal-specific genes *CaHWP1* and *CaALS3. Ca*Als3 is a

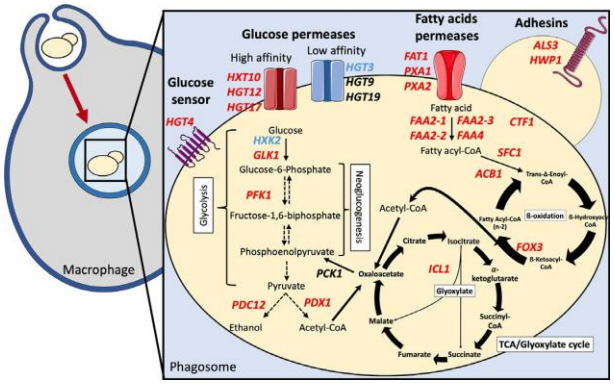

Figure 5. Schematic representation of *C. albicans* carbon metabolism after 30 min of phagocytosis in macrophages, according to key genes expression analysis. Over expressed genes are in red, under expressed genes are in blue and in black when the expression does not vary.

cell surface invasin-glycoprotein, belonging to the *C. albicans ALS* Family (Hoyer *et al.* 2008). *Ca*Hwp1 is a GPI-anchored cell wall protein (Nantel *et al.* 2002). Our data reveal a clear overexpression of both genes, by a factor of almost 5 for *CaALS3* and 2.3 for *CaHWP1* (Fig. 4). This indicates that our experimental conditions allow the activation of the yeast to hyphae transition program being part of the virulence factors associated to the metabolic profile of phagocytosed cells.

DISCUSSION

In this study, we sought to investigate the expression of carbon metabolism genes and more specifically glucose metabolism of *C. albicans* cells after 30 minutes of phagocytosis by macrophages (Fig. 5). Our findings reveal a clear over expression of the high-affinity glucose sensing and transport systems. *C. albicans* senses glucose via the SRR pathway (Brown, Sexton and Johnston 2006; Sexton, Brown and Johnston 2007; Brown, Sabina and Johnston 2009). *Ca*Hgt4, the high-affinity glucose sensor initiating this pathway is required for *CaHGT12*, *CaHXT10* and

CaHXT17 hexose transporter induction. Data from Brown, Sexton and Johnston (2006) revealed that in the absence of glucose (glycerol), the level of *CaHGT4*, *CaHGT12*, *CaHXT10* and *CaHXT17* did not exceed the threshold of detection, while at low glucose concentration (0.025–0.05%), the expression of all these genes was enhanced and clearly detected. Regarding our data, expression of the high-affinity sensor and transporters after 30 min of phagocytosis could suggest that traces of glucose initially present in the macrophages have been detected by the pathogen. Anyhow, an intracellular glucose concentration higher than 0.1% would lead to glucose repression and therefore under expression of high-affinity glucose permease genes (Laurian *et al.* 2019). However, Munoz *et al.* (2019) revealed the expression of several high-affinity hexose transporters, not only in infected macrophages but also in macrophages exposed to *C. albicans* that remain uninfected. This could suggest that over expression of high-affinity transporters could not be only a response to phagocytosis.

Among the transporters, *CaHGT12* is a particular case, especially in terms of its very high level of expression detected after 30 min of phagocytosis. This high-affinity transporter, previously known as *CaGS,* has an impact on filamentation because the *Cags* deletion mutant is hypofilamentous (Luongo, Porta and Maresca 2005; Luo, Tong and Farley 2007). When used to transform the hexose deficient strain of *Saccharomyces cerevisiae* EBY-F4–1, *CaGS* coding sequence restores the capacity of the mutant to grow on media containing glucose and fructose (Luo, Tong and Farley 2007). This transporter which has never been characterized, shows sequence similarities with glucose sensors in yeasts. However, its C-terminal cytoplasmic domain contains only 58 residues and no glucose sensor conserved motif, which is more consistent with a typical hexose transporter (Prigneau *et al.* 2003). Finally, its high level of expression inside the macrophage and its implication in yeast to hyphae transition suggest that *CaHGT12* expression could also be specifically governed by intracellular environmental conditions like anoxia, low pH or nutritional deficiency. The expression of this particular glucose transporter, required for growth at low glucose concentration (Brown, Sexton and Johnston 2006) could be a preliminary and necessary step to the further progress of *C. albicans* macrophage infection. Future research for an extensive characterization of this transporter might provide further insights on its specific role during the interaction.

While high-affinity glucose detection and transport systems are over expressed, transcripts of the hexokinase *CaHXK2* are clearly under represented (x0.54) and those of the glucokinase *CaGLK1* are expressed 1.25 times more in the macrophage than in the presence of glycerol. This shows that both genes do not respond to the same regulation signals in the macrophages and also that glucose induction of the hexokinase gene does not occur in this environment. Expression induction of glucokinase gene in the absence or presence of low concentrations of glucose is not totally surprising since it has been described in *C. albicans* and *S. cerevisiae* (Herrero *et al.* 1995; Laurian *et al.* 2019). The under expression of the hexokinase *CaHXK2* has already been revealed in a proteomic study of the interaction between *C. albicans* and macrophages conducted after 3 h of phagocytosis (Kitahara *et al.* 2015). Data presented in this work confirm our results and show that glucokinase was over expressed by a factor of 1.35 while the hexokinase was under detected by a factor of 0.58 during the interaction. Finally, comparison of our data to this proteomic study also suggest that there is not a great discrepancy between transcripts and protein detection under these conditions. Moreover, in a previous study, we have shown that SRR-dependent high-affinity transporters (*CaHGH12*, *CaHGT7*, *CaHXT10*) were under-expressed in a glucokinase mutant (Laurian *et al.* 2019). Thus, the expression of low-affinity glucose transporters is in accordance with a detectable *CaGLK1* expression level. The intracellular fate of *C. albicans* in a macrophage still remain partially unknown. The fungal pathogen must face the oxidative stress generated by reactive oxygen and nitrogen intermediates and low pH conditions in the phagolysosome (Erwig and Gow 2016; Viola *et al.* 2019). Morerover, hypoxic conditions might occur inside the macrophage. Previously, we have shown that glucokinases and hexokinases are differentially regulated by hypoxic conditions. Contrary to *CaHXK2*, the transcription of *CaGLK1* is strongly induced by hypoxia (Laurian *et al.* 2019). Moreover, we have shown that the hexokinase deletion mutant *Cahxk2* was hypo virulent in a macrophage model (Laurian *et al.* 2019). However, our ddPCR data show that *CaHXK2* is under expressed after 30 min of phagocytosis. This could suggest that *CaHXK2* is not necessary to the first step of infection (30 min) but in the later phases of the interaction. This is in accordance with the behavior of the *Cahxk2* mutant which appeared clearly hypovirulent only after 4 h of phagocytosis (Laurian *et al.* 2019).

Our data present a clear starvation-like response in which the alternative carbon metabolism is significantly overexpressed and takes place since the early step of phagocytosis. Activation of the transcription of genes implicated in the catabolic process of fatty acid β-oxidation (transport, transcription factors and enzymatic transformation) settles in within 30 min. The transcription of *CaPXA1* implicated in the import of longchain fatty acids in peroxisome, of *CaFAA2–3* and *CAFAA4,* Acyl-CoAsynthases (Tejima *et al.* 2018) and *CaSFC1* that could link fatty acid metabolism and gluconeogenesis (Zeman *et al.* 2016), is particularly activated. Even if the content of the phagolysosome has not been accurately analyzed, it could be particularly rich in lipids and their degradation products (Lorenz and Fink 2001; Lorenz, Bender and Fink 2004). Simple compounds derived from the breakdown of fatty acids via β-oxidation results in Acetyl-CoA, which use would require the glyoxylate cycle. Indeed, the activity of the glyoxylate cycle depends on a functional β-oxidation pathway in *C. albicans* (Piekarska *et al.* 2008). For generating C4 units, the glyoxylate cycle requires C2 compounds, provided by β-oxidation of fatty acids. This is consistent with our data that show the over expression of *CaICL1,* considered as a key step of the glyoxylic cycle. Many transcriptomic and proteomic studies attest the up regulation of the glyoxylate cycle during phagocytosis (Lorenz and Fink 2002; Barelle *et al.* 2006; Ferna´ndez-Arenas *et al.* 2007; Diez-Orejas and Ferna´ndez-Arenas 2008; Jime´nez-Lo´pez and Lorenz 2013; Kitahara *et al.* 2015). Surprisingly, *CaPCK1* expression level, a reference gluconeogenic gene, was not induced. This has already been observed during *C. albicans* phagocytosis by neutrophils (Barelle *et al.* 2006; Niemiec *et al.* 2017). In that case, *CaPCK1* expression level was lower than that of *CaICL1.* This could suggest that fatty acid metabolism and glyoxylate cycle activity are not necessarily connected to gluconeogenesis and hexose anabolism. This could also reveal that the transfer from glycerol to the macrophage does not induce overexpression of this gluconeogenic gene because its transcription could be already induced in glycerol medium.

To complete our study, we have considered the transcription of two genes encoding the hyphal proteins *CaALS3* and *CaHWP1.* To evaluate the impact of macrophage intracellular environment on gene expression, we have removed from the culture medium serum and glucose, considered as putative filamentation inducers. Our results indicate that induction of hyphae-associated

gene transcription occurs after all within 30 min of phagocytosis. Even if the intracellular conditions that could induce filamentation within the macrophage are not clearly defined, our data indicate that the environmental conditions met during the first steps of phagocytosis, induce the expression of genes implicated in adhesion and associated to the filamentous development of the pathogen.

Recently, Munoz *et al.* (2019) revealed the molecular heterogeneity within a population of macrophages encountering *C. albicans* cells. Bulk approaches measure the average transcriptional signal of millions of cells. Our experimental procedure may consider macrophage containing dead phagocytosed *C.albicans,* or infected by different number of cells. Moreover, if they were internalized with a delay, some yeast cells may have stayed less than 30 minutes in the macrophage. However, the first objective of this study was to show that ddPCR is an appropriate technique to detect *C. albicans* transcripts during an early step of phagocytosis and this method appears reproductible since the values obtained with the biological replicates are close. Although genes implicated of glucose metabolism were not clearly induced, genes involved in high-affinity glucose detection and transport were over expressed. We suggest that despite the low amount of glucose initially present in the macrophage that might be detected by the SRR glucose signaling pathway, phagocytosed *C. albicans* cells are clearly turned from the beginning towards the utilization of alternative carbon sources and notably lipids.

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSPD* online.

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Conflicts of interest. None declared.

REFERENCES

Almeida RS, Brunke S, Albrecht A *et al.* The hyphal-associated adhesin and invasin Als3 of *Candida albicans* mediates iron acquisition from host ferritin. *PLoS Pathog* 2008;**4**:e1000217.

Barelle CJ, Priest CL, Maccallum DM *et al.* Niche-specific regulation of central metabolic pathways in a fungal pathogen. *Cell Microbiol* 2006;**8**:961–71.

Bhat S, Herrmann J, Armishaw P *et al.* Single molecule detection in nanofluidic digital array enables accurate measurement of DNA copy number. *Anal Bioanal Chem* 2009;**394**:457–67.

Biswas S, Van Dijck P, Datta A. Environmental sensing and signal transduction pathways regulating morphopathogenic determinants of *Candida albicans. Microbiol Mol Biol Rev* 2007;**71**:348–76.

Brown AJP, Brown GD, Netea MG *et al.* Metabolism impacts upon *Candida* immunogenicity and pathogenicity at multiple levels. *Trends Microbiol* 2014;**22**:614–22.

Brown V, Sabina J, Johnston M. Specialized sugar sensing in diverse fungi. *Curr Biol* 2009;**19**:436–41.

Brown V, Sexton JA, Johnston M. A glucose sensor in *Candida albicans. Eukaryot Cell* 2006;**5**:1726–37.

Cannon GJ, Swanson JA. The macrophage capacity for phagocytosis. *J Cell Sci* 1992;**101**:907–13.

Dementhon K, El-Kirat-Chatel S, Noe¨ l T. Development of an in vitro model for the multi-parametric quantification of the cellular interactions between *Candida* yeasts and phagocytes. *PLoS One* 2012;**7**:e32621.

Deshmukh RA, Joshi K, Bhand S *et al.* Recent developments in detection and enumeration of waterborne bacteria: a retrospective minireview. *Microbiologyopen* 2016;**5**:901–22.

Diez-Orejas R, Ferna´ndez-Arenas E. *Candida albicans*-macrophage interactions: genomic and proteomic insights. *Future Microbiol* 2008;**3**:661–81.

Erwig LP, Gow NAR. Interactions of fungal pathogens with phagocytes. *Nat Rev Microbiol* 2016;**14**:163–76.

Fan J, Chaturvedi V, Shen S-H. Identification and phylogenetic analysis of a glucose transporter gene family from the human pathogenic yeast *Candida albicans. J Mol Evol* 2002;**55**:336–46.

Ferna´ndez-Arenas E, Cabezo´n V, Bermejo C *et al.* Integrated proteomics and genomics strategies bring new insight into *Candida albicans* response upon macrophage interaction. *Mol Cell Proteomics* 2007;**6**:460–78.

Herrero P, Gal´ındez J, Ruiz N *et al.* Transcriptional regulation of the *Saccharomyces cerevisiae* HXK1, HXK2 and GLK1 genes. *Yeast* 1995;**11**:137–44.

Hoyer LL, Green CB, Oh S-H *et al.* Discovering the secrets of the *Candida albicans* agglutinin-like sequence (*ALS*) gene family–a sticky pursuit. *Med Mycol* 2008;**46**:1–15.

Hudson DA, Sciascia QL, Sanders RJ *et al.* Identification of the dialysable serum inducer of germ-tube formation in *Candida albicans*. *Microbiology (Reading, Engl)* 2004;**150**:3041–9.

Jime´nez-Lo´pez C, Lorenz MC. Fungal immune evasion in a model host-pathogen interaction: *Candida albicans* versus macrophages. *PLoS Pathog* 2013;**9**:e1003741.

Kitahara N, Morisaka H, Aoki W *et al.* Description of the interaction between *Candida albicans* and macrophages by mixed and quantitative proteome analysis without isolation. *AMB Express* 2015;**5**:127.

Laurian R, Dementhon K, Doume`che B *et al.* Hexokinase and glucokinases are essential for fitness and virulence in the pathogenic yeast *Candida albicans. Front Microbiol* 2019;**10**:327.

Lorberg A, Kirchrath L, Ernst JF *et al.* Genetic and biochemical characterization of phosphofructokinase from the opportunistic pathogenic yeast *Candida albicans. Eur J Biochem* 1999;**260**:217–26.

Lorenz MC, Bender JA, Fink GR. Transcriptional response of *Candida albicans* upon internalization by macrophages. *Eukaryot Cell* 2004;**3**:1076–87.

Lorenz MC, Fink GR. The glyoxylate cycle is required for fungal virulence. *Nature* 2001;**412**:83–6.

Lorenz MC, Fink GR. Life and death in a macrophage: role of the glyoxylate cycle in virulence. *Eukaryot Cell* 2002;**1**:657–62.

Luo L, Tong X, Farley PC. The *Candida albicans* gene *HGT12* (orf19.7094) encodes a hexose transporter. *FEMS Immunol Med Microbiol* 2007;**51**:14–7.

Luongo M, Porta A, Maresca B. Homology, disruption and phenotypic analysis of *CaGS Candida albicans* gene induced during macrophage infection. *FEMS Immunol Med Microbiol* 2005;**45**:471–8.

Montenegro DA, Borda LF, Neuta Y *et al.* Oral and uro-vaginal intra-amniotic infection in women with preterm delivery: a case-control study. *J Investig Clin Dent* 2019;**10**:e12396.

Munoz JF, Delorey T, Ford CB *et al.* Coordinated host-pathogen transcriptional dynamics revealed using sorted subpopulations and single macrophages infected with *Candida albicans*. *Nat Commun* 2019;**8**:1607.

Nantel A, Dignard D, Bachewich C *et al.* Transcription profiling of *Candida albicans* cells undergoing the yeast-to-hyphal transition. *Mol Biol Cell* 2002;**13**:3452–65.

Niemiec MJ, Grumaz C, Ermert D *et al.* Dual transcriptome of the immediate neutrophil and *Candida albicans* interplay. *BMC Genomics* 2017;**18**:696.

Noble SM, Gianetti BA, Witchley JN. Candida albicans cell-type switching and functional plasticity in the mammalian host. *Nat Rev Microbiol* 2017;**15**:96–108.

Odds FC. *F. C. Odds, Candida and Candidosis, A Review and Bibliography (Second Edition). X + 468 S., 97 Abb., 92 Tab. u. 22 Farbtafeln. London—Philadelphia—Toronto—Sydney—Tokyo 1988. Baillie`re Tindall (W. B. Saunders). ISBN: 0–7020–1265–3.*, 1988.

O'Meara TR, Cowen LE. Insights into the host-pathogen interactions: *C. albicans* manipulation of macrophage pyroptosis. *Microbial Cell* 2018*;* **5**:566–8.

Piekarska K, Hardy G, Mol E *et al.* The activity of the glyoxylate cycle in peroxisomes of *Candida albicans* depends on a functional beta-oxidation pathway: evidence for reduced metabolite transport across the peroxisomal membrane. *Microbiology (Reading, Engl)* 2008;**154**:3061–72.

Prigneau O, Porta A, Poudrier JA *et al.* Genes involved in beta-oxidation, energy metabolism and glyoxylate cycle are induced by *Candida albicans* during macrophage infection. *Yeast* 2003;**20**:723–30.

Ramirez MA, Lorenz MC. The transcription factor CTF1 regulates beta-oxidation in *C. albicans*. *Eukaryot Cell* 2009;**8**:1604–14.

Romani L. Immunity to fungal infections. *Nat Rev Immunol* 2011;**11**:275–88.

Segin F, Le Pape A. 31P and 13C nuclear magnetic resonance studies of macrophages. *Immunomethods* 1994;**4**:179–87.

Sexton JA, Brown V, Johnston M. Regulation of sugar transport and metabolism by the Rgt1 transcriptional repressor. *Yeast* 2007;**24**:847–60.

Talarico S, Safaeian M, Gonzalez P *et al.* Quantitative detection and genotyping of *Helicobacter pylori* from stool using droplet digital PCR reveals variation in bacterial loads that correlates with cagA virulence gene carriage. *Helicobacter* 2016;**21**:325–33.

Tejima K, Ishiai M, Murayama SO *et al. Candida albicans* fatty acyl-CoA synthetase, *Ca*Faa4p, is involved in the uptake of exogenous long-chain fatty acids and cell activity in the biofilm. *Curr Genet* 2018;**64**:429–41.

Tucey TM, Verma J, Harrison PF *et al.* Glucose homeostasis is important for immune cell viability during *Candida* challenge and host survival of systemic fungal infection. *Cell Metab* 2018;**27**:988–1006.e7.

Uwamahoro N, Verma-Gaur J, Shen H-H *et al.* The pathogen *Candida albicans* hijacks pyroptosis for escape from macrophages. *MBio* 2014;**5**:e00003–00014.

Viola A, Munari F, Sanchez-Rodriguez R *et al.* The metabolic signature of macrophage responses. *Front Immun* 2019;**10**:1462

Zeman I, Neboha´c˘ova´ M, Ge´recova´ G *et al.* Mitochondrial carriers link the catabolism of hydroxyaromatic compounds to the central metabolism in *Candida parapsilosis. G3 (Bethesda)* 2016;**6**:4047–58.