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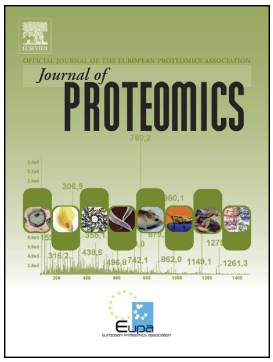

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The external face of *Candida albicans*: a proteomic view of the cell surface and the extracellular environment

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ABSTRACT

The cell surface and secreted proteins are the initial points of contact between *Candida albicans* and the host. Improvements in protein extraction approaches and mass spectrometers have allowed researchers to obtain a comprehensive knowledge of these external subproteomes. In this paper, we review the published proteomic studies that have examined *C. albicans* extracellular proteins, including the cell surface proteins or surfome and the secreted proteins or secretome. The use of different approaches to isolate cell wall and cell surface proteins, such as fractionation approaches or cell shaving, have resulted in different outcomes. Proteins with N-terminal signal peptide, known as classically secreted proteins, and those that lack the signal peptide, known as unconventionally secreted proteins, have been consistently identified. Existing studies on *C. albicans* extracellular vesicles reveal that they are relevant as an unconventional pathway of protein secretion and can help explain the presence of proteins without a signal peptide, including some moonlighting proteins, in the cell wall and the extracellular environment. According to the global view presented in this review, cell wall proteins, virulence factors such as adhesins or hydrolytic enzymes, metabolic enzymes and stress related-proteins are important groups of proteins in *C. albicans* surfome and secretome.

Biological Significance

Candida albicans extracellular proteins are involved in biofilm formation, cell nutrient acquisition and cell wall integrity maintenance. Furthermore, these proteins include virulence factors and immunogenic proteins. This review is of outstanding interest, not only because it extends knowledge of the *C. albicans* surface and extracellular proteins that could be related with pathogenesis, but also because it presents insights that may facilitate the future development of new antifungal drugs and vaccines and contributes to efforts to identify new biomarkers that can be employed to diagnose candidiasis. Here, we list more than 570 *C. albicans* proteins that have been identified in extracellular locations to deliver the most extensive catalogue of this type of proteins to date. Moreover, we describe 16 proteins detected at all locations analysed in the works revised. These proteins include the glycophosphatidylinositol (GPI)-anchored proteins Ecm33, Pga4 and Phr2 and unconventional secretory proteins such as Eft2, Eno1, Hsp70, Pdc11, Pgk1 and Tdh3. Furthermore, 13 of these 16 proteins are immunogenic and could represent a set of interesting candidates for biomarker discovery.

1. Introduction.

Candida albicans is an opportunistic fungus that can cause both mucosal and invasive infections. Invasive candidiasis constitutes a life-threatening condition in immunocompromised and critically ill patients [1]. As the external face, the cell wall is the first point of contact between *C. albicans* and the host. It plays a significant role in the shape and physical strength of the cell and its ability to colonise human tissues. The cell wall of *C. albicans* is a layered structure that is composed of β-1,3- and β-1,6-glucan, chitin, mannan and proteins [2, 3]. Variations in these components have a direct impact on the behaviour of yeast; for example, growth or cell attachment and recognition by the host [4]. Chaffin presented in-depth insights into cell wall proteins some years ago [5]. Of the proteins that have been investigated, some are adhesins, such as members of the Als family or Hyr1, and others are involved in virulence. The secreted proteins of *C. albicans* also play a pivotal role in virulence. These include secreted hydrolytic enzymes of different families such as secreted aspartyl proteases (Sap), lipases (Lip) and phospholipases (Plb) [5-8].

C. albicans surface and secreted proteins reach their final location after being secreted. The proteins that are transported through the classical secretory pathway carry a hydrophobic N-terminal signal sequence that is responsible for directing them into the endoplasmic reticulum (ER). They are transported through a complex system of internal vesicles through the Golgi apparatus to the plasma membrane where transmembrane proteins and glycophosphatidylinositol (GPI)-anchored proteins can be retained or continue to become covalently attached to the cell wall or secreted into the extracellular medium. The soluble secretory proteins are released into the periplasmic region from where they are diffused to the media [9-11]. In addition, *C. albicans*

proteins that lack a signal peptide have been identified on cell surface and in extracellular media and some unconventional secretory pathways of proteins have been proposed [12-16].

This paper presents an overall view of the proteomic studies that have previously analysed the surface and secretome, including extracellular vesicles (EVs) of *C. albicans* in terms of both the proteomic approaches and the results and insights obtained. It also highlights recent findings related to *C. albicans* EVs and their role in protein secretion.

2. Proteomic analysis of *C. albicans* cell wall and surface proteins

a) The diversity of *C. albicans* cell wall proteins

The cell wall proteins that are covalently attached to cell wall polysaccharides are linked in different ways. The most abundant proteins are linked to β-1,6-glucan through a GPI remnant (GPI-anchored proteins). Proteins with internal repeats (Pir) are linked directly to the β-1,3-glucan. The third group of proteins, the cell-associated proteins, lack a covalent attachment to the polysaccharide matrix [5]. The diversity of anchorages makes the extraction of proteins from the polysaccharide matrix difficult.

The first proteomic attempt to identify yeast cell wall proteins, and more specifically the proteins involved in yeast cell wall biogenesis, was a study on the proteins secreted by regenerating protoplasts in *S. cerevisiae* [17, 18]. The approach employed in the research avoided extracting proteins from the cell wall. It was found that upon incubation of the protoplasts in regenerating conditions numerous proteins were secreted into the culture medium, including structural cell wall proteins and the enzymes involved in cell wall biogenesis, making them easy to collect and analyse. This work describes a 2D-PAGE analysis that allowed the construction of a reference map including 32 different proteins identified, firstly by Edman degradation or immunoblotting and then by mass spectrometry. The proteins corresponded to cell wall proteins such as Hsp150

or Ydr055w/Pst1 for protoplast secreted protein1 (a homologue of Ecm33), cell wall hydrolytic enzymes (i.e., Bgl2), glycolytic enzymes (Eno2 or Fba1) and heat shock proteins. These types of proteins were consistently identified in the other cell wall proteomic studies described below. However, the identification of proteins without a signal peptide at the extracellular environment, such as glycolytic enzymes, was very controversial at that time, despite the fact that genetic evidence supported this finding [17]. In *C. albicans,* some studies with proteins secreted by regenerating protoplasts were also carried out, but they were more focused on the identification of immunogenic proteins than in the description of the cell wall proteome [19, 20]. In the first work of this nature, Pitarch et al. detected 4 immunoreactive proteins by 2D-PAGE and Western blotting with sera from patients with systemic candidiasis using proteins secreted by yeast protoplasts during cell wall regeneration [19]. The most reactive proteins were enolase and a 34-kDa protein. In a more recent work that employed the same approach, it was concluded that serum anti-Bgl2 antibodies represent accurate diagnostic biomarkers of systemic candidiasis and serum anti-wall enolase antibodies are a prognostic indicators of this infection [20].

b) Analysis of proteins from isolated cell walls.

In several studies, the authors **isolated cell walls** after cell breakage to extract and identify *C. albicans* cell wall proteins via different methodologies (Figure 1, cw1, cw2 and cw3). Pitarch et al. used a **sequential method** to solubilise the cell wall proteins according to their anchors to the cell wall polysaccharides from yeast and hyphae (Figure 1, cw1) [21]. The extracted proteins were separated by **2D-PAGE** and identified by peptide mass fingerprinting (PMF) with **MALDI-TOF** or sequencing analysis by MALDI-TOF/TOF. SDS and DTT treatments were carried out to extract non-covalently cell surface proteins followed by extraction either by mild alkali conditions (to extract Pir proteins) or by enzymatic treatment with glucanases and

chitinases (to release other proteins anchored to the cell wall glucan and chitin, including GPI proteins). The 2D reference map of each of these fractions was established and several proteins, predominantly those of the SDS-DTT extracted fraction, were identified. They included proteins from similar categories to those described in the previous study: known cell wall proteins, such as Hsp150, one of the Phr proteins (GPI anchored) or cell wall hydrolytic enzymes (i.e., Bgl2) and cell wall-associated proteins that lacked signal peptide. The last group comprised glycolytic enzymes, heat shock proteins and elongation factors, some of which are classified as multifunctional or moonlighting proteins because they are located in more than one cellular compartment and perform different functions [16]. It is important to point out that most of the proteins present in the fractions extracted with glucanases or chitinase were highly glycosylated; thus, it was very difficult to separate and identify them by 2D-PAGE and PMF analysis respectively as a lot of peptides are highly modified.

To avoid the limitations of 2D-PAGE and PMF, some studies have employed an **LC-MS/MS** based strategy for the analysis of **proteins extracted** from *C. albicans* isolated cell walls (Figure 1, cw2) [22, 23]. Within these studies, the isolated walls were treated with hydrogen fluoride-pyridine (HF-pyridine) (to release GPI proteins), SDS or β-mercaptoethanol (for proteins bound by disulphide bridges), or NaOH (for proteins bound by alkali-labile ester linkages as the ALS cell wall proteins). Up to 19 GPI-anchored proteins and 10 non-GPI cell wall proteins bearing signal peptide were identified in these works as well as 4 membrane proteins and 66 proteins without signal peptide [22, 23]. To selectively identify the proteins attached to cell wall polysaccharides through covalent binding, other authors have previously described HF-pyridine, NaOH and glucanase treatments of isolated cell walls from *C. albicans* after stringent washing

and hot reducing agent treatment in SDS extraction buffer, resulting in the identification of 14 proteins by LC-MS/MS [24].

As cell wall proteins are difficult to extract, a different strategy based on **the trypsin digestion of the isolated cell walls** (Figure 1, cw3) after stringent washing and treatment with a hot reducing agent was employed to identify only covalently bound proteins. The released peptides were then analysed by LC-MS/MS. The works that employed this strategy also included ^{15}N-metabolic labelling of reference cell walls for the relative quantification of the dynamics of the identified proteins in different *in vitro* growing conditions [25-30]. Around 20 covalently bound cell wall proteins were relatively quantified under most conditions, most of which were GPI-anchored proteins. The non-covalently bound cell wall-associated proteins could not be identified since the authors discarded them before trypsin digestion [27]. This strategy generated interesting insights into the study of the dynamics of the GPI and other covalently bound cell wall proteins; however, it does not represent an adequate approach by which a global view of the whole fungal cell wall proteomes of *C. albicans* can be obtained.

c) Analysis of the cell surface proteome or "surfome": the cell shaving approach

Another strategy by which it is possible to identify the *C. albicans* proteins exposed to the host is through the analysis of the surface proteins of cells. To identify these, Urban et al. labelled the yeast and hyphal surface proteins with a membrane-impermeable biotin derivative that allowed their subsequent purification after breaking the cells, describing that Tsa1 was localised only in hyphae cell surface [14]. More recently, other works have analysed the surface proteins without breaking the cells. Martinez-Gomariz et al. analysed the non-covalently attached cell-surface proteins from biofilm and planktonic cells extracted with ammonium carbonate and β-mercaptoethanol buffer by **2D-DIGE (differential in-gel electrophoresis)** (figure 1, sf1) [31].

Twenty-five proteins, most of which were non-classically secreted proteins, were identified and differences in abundance among the three growing forms were highlighted.

Taking into account the difficulties of extraction and the 2D-PAGE separation of the covalently anchored cell wall proteins, more recently, a non-gel proteomic strategy based on **live cell shaving** by proteases and LC-MS/MS (Figure 1, sf2) has been employed in several works. This method was developed as a means of identifying vaccine candidates for the group A *Streptococcus* and has been widely tested as a method with the aim of identifying the cell surface proteins of several microorganisms [32, 33]. In fungi, the trypsin shaving of the live cells should release peptides from the cell wall proteins or the extracellular domains of cytoplasmic membrane proteins. Therefore, this represents a fast and efficient strategy that can be employed to identify the fungi surface proteins (surfome) recognised by the host.

Insenser et al. conducted a global study to identify *Saccharomyces cerevisiae* cell surface proteins from intact cells that combined two of the strategies described above: a) The release of loosely associated or S-S linked wall proteins by treatment with dithiothreitol followed by 2D-PAGE and MS, and b) trypsin shaving followed of LC–MS/MS [34]. Proteins from different cellular processes, such as cell wall organisation, cell rescue, protein fate or metabolism, were identified via both strategies; however, a higher number of cell wall remodelling enzymes and GPI proteins were obtained with the gel-free approach. In total more proteins were identified by the gel-based strategy than by gel-free proteomics and only 20% of the proteins were common to both approaches, highlighting the need to employ several strategies to acquire a complete knowledge of the cell surface proteome.

The analysis of the surfome of *C. albicans* by **trypsin shaving** and MALDI-TOF/TOF was reported first in yeast cells and later in a larger study that included yeast cells, hyphae and

biofilms that were also subjected to cell shaving [35, 36]. Up to 131 proteins were identified in the second work, and these included relevant cell wall proteins that had not been identified in the 2D-DIGE analysis of β-mercaptoethanol-extracted proteins of similar yeast cells, hyphae and biofilms, revealing the utility of this fast and easy strategy [31]. More recently, Gil-Bona et al. published research that described the tryptic digestion of live yeast cells and hyphae followed by LC-MS/MS methodology, which identified more than 400 and 900 cell surface proteins in yeast and hyphae cells respectively [37]. Many more proteins were identified than those detected in previous works due to the improvement in the sensitivity of the mass spectrometer, an LTQ-Orbitrap Velos ultra-high resolution mass analyser, that was used in this work. The total number of proteins identified in each category was higher than that identified in previous studies: close to 30 GPI-anchored proteins and more than 50 cell wall organisation or biogenesis-related proteins. Furthermore, a relevant number of plasma membrane proteins and some adhesion-related proteins, such as members of the Als family, Sim1 or Msb2, were identified. This work was supplemented with a functional analysis of selected mutant strains of proteins with unknown function that allowed the description of proteins involved in relevant cellular processes such as osmotic and oxidative stress resistance (e.g., Ali1 and Mci4), cell wall maintenance (e.g., Tos1 or Orf19.5352) or host-pathogen interaction (e.g., Orf19.3060 and Pst3). Marin et al. published a complementary work based on this shaving strategy that aimed to simultaneously identify *C. albicans* surface proteins and the human serum proteins coating fungi cells simultaneously [38]. Using an LTQ-Orbitrap Velos, in excess of 200 human proteins and close to 400 *C. albicans* proteins were identified, including 23 GPI-anchored proteins, 36 plasma membrane proteins and 52 proteins that had been previously described as immunogenic, such as Bgl2, Eno1, Hsp90 and Met6 [20, 39-42]. Some of the plasma membrane proteins identified in both the

work of Gil-Bona et al. and Marin et al., such as Pma1, had been previously reported in a plasma membrane proteome analysis [43]. In this work, the proteomic analysis of plasma membrane proteins (including GPI-anchored proteins) was conducted to test the efficiency of different plasma membrane enrichment methods and different mass spectrometers after the formation of *C. albicans* protoplast (Figure 1). The detection of some of these plasma membrane proteins in the surfome analysis was not unexpected. The commented works of Gil-bona et al. and Marin et al. which used the shaving approach, provided the most comprehensive descriptions of *C. albicans* yeast and hyphal surface proteins, with the largest number of cell wall-related proteins identified [37, 38]. Moreover, in Marin et al.'s work, the growth media included serum to simulate conditions similar to those found physiologically.

3. Secreted proteins

As proteins secreted through the classical secretory pathway have an N-terminal signal peptide, this signal can be used to predict the mechanism of secretion. The predicted secreted proteins of *C. albicans* have been described in some studies. In 2003, Lee et al. described a computationally-predicted *C. albicans* secretome that consisted of up to 283 ORFs [44]. More recently, the Fungal Secretome Knowledge Base was constructed, and an assessment of only the proteins with N-terminal signal peptide indicated that 3.1% of the *C. albicans* proteome was secreted (449 proteins) [45].

A genetic screening that identified N-terminal signal sequences was published several years ago. It provides an overview of the *C. albicans* putatively exported proteins [46]. This approach employed in-frame fusions of *C. albicans* genes with an intracellular allele of the invertase gene of *S. cerevisiae*, *SUC2*, as a reporter. The analysis resulted in the identification of 83 clones, including enzymes involved in cell wall synthesis, in different morphological transitions and in

protein secretion, as well as membrane receptors and transporters. In addition, eleven *C. albicans* ORFs homologous to unknown or putative proteins and five that encoded novel secreted proteins without known homologues in other organisms at that time were identified, obtaining a global view of what *C. albicans* secretes to survive.

a) Proteomic analysis of whole secretome

More recently, different proteomic approaches (Figure 1, sc1, sc2 and sc3) have been used to obtain a global knowledge of the extracellular proteins or "secretome" of *C. albicans* under several growing conditions by identifying different sets of proteins. Some works are based on the concentration of the **whole cultured medium** free of cells followed by different methods of protein separation. While Sorgo et al. and Ene et al. studied the secretome under different pH, temperatures and carbon sources using a MS-based methodology (Figure 1, sc1), Luo et al. combined 2D-PAGE (Figure 1, sc2) and LC-MS/MS (Figure 1, sc1) to study the yeast-to-hypha transition [15, 26, 47]. A miscellaneous group of proteins were identified in these studies that included secretory proteins (e.g., members of the Als or Sap families), and cell wall-related proteins (e.g., Cht1, Cht2, Cht3, Phr1 or Phr2). The results of some of these proteomic works, as well as the importance of the *C. albicans* secretome for virulence and its adaptation in response to environmental conditions, has been previously revised [48, 49].

The total number of proteins identified in the proteomic works described above increased from close to 80 identified in Sorgo et al. to 101 and 410 proteins identified in Luo et al. in yeast cells and hyphae, respectively. Furthermore, 19 immunogenic proteins were identified in the latter study of *C. albicans* secretome, including the exo-glucanase Xog1 and the glycolytic enzymes Eno1 and Fba1 [47]. Besides the classically secreted proteins, a relevant proportion of proteins that lack signal peptide have been detected in the extracellular media of *C. albicans* (e.g., Cyp5,

Eno1 or Tdh3) [15, 47]. These proteins were involved in stress responses, transport, translation and carbohydrate metabolic processes and were originally characterised as cytoplasmic; however, some of them have also been detected in the plasma membrane or, as commented above, in the cell wall [5, 13, 43, 50, 51]. As they do not possess a signal peptide, it is feasible that these proteins reach the cell surface via a non-conventional secretion route [2, 16, 52, 53]. In fact, a different database of predicted secreted fungal proteins, the Fungal Secretome Database, has been developed. Because a program to predict whether a protein is secreted by non-classical pathways was employed to construct this database, it includes proteins with and without N-terminal signal peptide. It lists around 800 proteins in the *C. albicans* putative secretome [54]. As the mechanisms of unconventional secretion are currently not totally clear, these data have to be interpreted carefully.

b) Proteomic analysis of secretome separated into extracellular vesicles and vesicles-free fractions.

A deeper proteomic study of *C. albicans* secretome included a fractionation step in the process by which the extracellular medium was separated by ultracentrifugation of the EVs from the rest of the secretome (EV-free supernatant) before the LC-MS/MS analysis (**secretome fractionation,** Figure 1, sc3) [55]. A similar study was previously performed on *Paracoccidioides brasiliensis* [56]. In the *C. albicans* study, 61 proteins were identified in the EV-free supernatant of yeast cells grown at 30 ºC, of which more than 90% were classical secretory proteins with N-terminal signal peptide. This fraction was enriched in cell wall (49%) and secreted pathogenesis-related proteins (21%). Among them, hydrolytic enzymes and adhesins, members of the most important families of secreted proteins, such as Sap2, Sap3 or Als4, were exclusively detected in this EV-free secretome fraction. The classically cytoplasmic

proteins or unconventionally secreted proteins that have also been identified in the previously described secretomes and in the cell surface analysis (i.e., Eno1 and Tdh3) were exclusively identified in EVs (data thoroughly discussed in the next section). In addition, 7 proteins were identified in the EVs that have never previously been described in *C. albicans* yeast secretome: Axl2, Dfg5, Orf19.31, Orf19.7596, Plb1, Rax2 and Sap99. Thus, the separation of EVs from the rest of the secretome rendered interesting data and enhanced existing knowledge of the *C. albicans* secretome.

The total number of proteins identified by Gil-Bona et al. through a combination of EV-free supernatant and EVs analysis was 96 [55]. This number was closer to that obtained during Luo et al.'s work on yeast growing cells samples (101 proteins). In this work, the EVs were not separate from the rest of the secretome and, presumably, EV proteins were also identified [47]. More than half of the proteins detected in the yeast (61 proteins) were common to both of these studies. These common proteins include the Sap family members (Sap2, Sap3, Sap7 and Sap9), Als family members (Als2 and Als4) and many cell wall-related proteins (e.g., Bgl2, Cht1-3, Ecm33, Pga4, pga45, Pga52). The detection of different proteins in the respective studies could be attributed to differences in the nutrient acquisition or temperature adaptation but also due to the use of 2D gel analysis used by Luo et al., as 7 out of the 8 proteins that were detected only by the gel-based approach in Lou et al.'s work were not detected in the study conducted by Gil-Bona et al. [47, 55].

4. Extracellular vesicles (EVs) as carriers of secreted proteins

a) Proteomic analysis of *C. albicans* extracellular vesicles

Among the non-conventional mechanisms of protein secretion that have been described in the literature, one possible explanation for these proteins lacking signal peptide at the cell surface

and the extracellular media is that they were transported by EVs. These compartments, which are secreted from mammalian to bacteria cells, were first described in *Cryptococcus neoformans* fungi and later characterised in other fungi, including *C. albicans* [55-66]. They carry diverse proteins, lipids, polysaccharides, pigments and RNA. The studies on *C. albicans* that have been published to date have found that these structures transport different proteins, lipids and RNA and are of different sizes, ranging from less than 50 nm to more than 850 nm [55, 65, 66]. As discussed earlier, a comparative proteomic analysis of the extracellular vesicles and the soluble secreted proteins from *C. albicans* strain SC5314 grown at 30 ºC in Synthetic Defined (SD) media over 16 hours showed that most of the secreted proteins that lack signal peptide are transported to the extracellular environment by the EVs, and the majority of them are not present in the EV-free supernatant, suggesting that vesicular transport might be the major route of the extracellular delivery, at least in the studied conditions [55]. Among these unconventionally secreted proteins, the exocytosis and endocytosis-related proteins and metabolic-related proteins, including proteins classically defined as moonlighting proteins, were identified only in the EVs sample. Within this category, proteins like the enolase (Eno1), the glyceraldehyde-3-phosphate dehydrogenase (Tdh3) or the heat-shock proteins (Hsp70), among others, were detected in the EVs. In addition, classically secreted proteins, such as Sap family members or Plb family members, were identified in the EVs, as well as transmembrane proteins (e.g., Fet3, Fet34 or Pma1) and the cell wall proteins (e.g., Bgl2, Ecm33, Mp65 or Tos1).

The protein composition of the EVs from other *C. albicans* clinical isolates different from SC5314 (11 and ATCC 90028) growing at the same temperature were also analysed [65]. In this case, the EVs were obtained from 48-hour Sabouraud cultures and the proteins identified were also related to cell wall, cytoplasm, extracellular environment and plasma membrane. Only 13

proteins identified in these two strains were also identified in SC5314 EVs grown at 30 °C, corresponding membrane proteins (Msb2), cell wall related proteins (Bgl2, Cht2, Cht3, Mp65, Phr2, Scw11, Sim1 and Tos1), and unconventionally secreted proteins (Eno1, Tdh3, Gmp1, and Pdc11) (Figure 2a).

EVs from the *C. albicans* strain SC5314 under different growth conditions were also analysed [66]. This study examined the differences between the composition of EVs in SC5314 and three mutants in genes involved in phospholipid biosynthesis from three-day-old rich medium culture (37 °C, 72 hours, YPD medium). The number of proteins identified in the parental strain was much higher at 37 °C in rich medium than it was at 30 °C in SD, 164 and 75 proteins respectively, corresponding to 214 unique proteins (Figure 2b). Only 11% of the total of proteins identified in SC5314 (25) were observed under both conditions. Importantly, these common proteins included cell wall-related proteins and several proteins that lacked the signal peptide involved in metabolism (e.g., Eno1, Pgk1, Tdh3 or Ald5), stress response proteins (e.g., Hsp70) and protein synthesis (Eft2). Furthermore, some transmembrane proteins, such as the glucan synthase Gsc1, or transported proteins were identified in the EVs (Figure 2b). Even though the majority of the proteins are not shared, proteins identified only at 37 °C corresponded mainly to the same groups of proteins without signal peptide, such as metabolic proteins or those involved in protein synthesis, among others. These analyses show that different growth conditions determine the protein content of the EVs of *C. albicans.* In addition to the different temperatures, these experiments employed different mediums and culture times. These variations influence the phase of growth and the needs for survival and maintenance, being different in a 72 hours' culture (presumably in stationary phase) than in a fresh culture in exponential phase. More stress response-related proteins were identified in the older culture (e.g., Hsp60, Hsp90, Ssb1 or Ssc1),

while cell wall and transporter proteins were more abundant in the fresh culture (e.g., Fet34, Fet99, Zrt2, Cht2 or Cht3). When the data from the different proteomic analyses of EVs of *C. albicans* wild-type strains in different conditions is combined, the total number of proteins identified to date is over 230 proteins, of which only 6 are common across all studies: Eno1, Gpm1, Pdc11, Phr2, Sim1 and Tdh3 (Figure 2c) [55, 65, 66].

b) Putative functions and biogenesis mechanisms of EVs

The total number of 230 proteins identified in all proteomic studies of *C. albicans* EVs is similar to the number of proteins identified in the EVs of other fungi such as *Histoplasma capsulatum* or *S. cerevisiae* [56, 58-60]. In the same sense, EVs have been described as carriers of proteins without signal peptide in different fungal species. Previous studies on *P. brasiliensis* that have separated EVs and EV-free secretomes have found that a higher proportion of EVs than EV-free secretome proteins were predicted as non-secretory, data in common with other three yeast EVs, *C. neoformans*, *H. capsulatum* and *S. cerevisiae* [56]. Another study on *S. cerevisiae* showed that some of the proteins without signal peptide that were detected in the extracellular media were transported by the EVs, as SDS treatment of the culture media entailed that some proteins associated with vesicles, such as Tdh1, Pdc1 or Pgk1, were detected in supernatant fraction, probably due to the membrane disruption [67]. However, the function of these proteins outside the *S. cerevisiae* cells is unknown.

The most recent reviews to analyse the EVs in fungi and other microorganisms propose different functions for these EVs related to the variety of molecules carried, including proteins, nucleic acids and polysaccharides. The proposed functions include concentration of secreted proteins, immunobiological activity or/and virulence [64, 68, 69]. In mammalian cells, EVs have been characterised into three different classes according to their size, cargo and pathway of origin:

exosomes, microvesicles and apoptotic bodies. In particular, exosomes are lipid bilayer structures that are smaller than 100 nm that have several relevant functions, such as intercellular communication, compound exchange, antigen presentation to mediate disease progression and other pathogenic and protective roles [70, 71]. Thus, the interest in characterising the molecular cargo of these vesicles has resulted in the development of the Exocarta, a manually curated web-based compendium of exosomal proteins from more than 10 different species [72]. On this website, we can find the list of the top 25 proteins that are most frequently identified in exosomes [72]. This list includes heat shock proteins, enolase, glyceraldehyde-3-phosphate dehydrogenase and membrane proteins, among others, that were also detected in *C. albicans* EVs. This indicates that there are some similarities between fungal and mammalian EVs. Some of these proteins are highly immunogenic in *C. albicans*, such as Eno1 or Hsp70, in the same way as other cell wall proteins such as Bgl2 or Mp65 [20, 47, 73, 74]. Therefore, it is not surprising that proteins derived from *C. albicans* EVs reacted with sera from *C. albicans*-infected mice and patients with systemic candidiasis. More data to support the idea that EVs play a role in the host immunity response are the stimulation of cytokines production after the internalisation by macrophages and dendritic cells of EVs and the reduction of the number of recovered viable yeasts from *Galleria mellonella* larvae inoculated with EVs before a *C. albicans* challenge in comparison with infected larvae used as control [55, 65]. This evidence suggests that *C. albicans* EVs play a role in host-pathogen communication. They also might play other significant roles also described for other fungal EVs [69, 75, 76]. Furthermore, six unconventionally secreted proteins carried by the EVs (Adh1, Eno1, Fba1, Pgk1, Tdh3 and Tsa1) are able to bind plasminogen, which is also related to the host interaction [77]. These moonlighting proteins have a metabolic or antioxidant intracellular function and a binding role

outside the cell. Therefore, the fact that EV transports them reinforces the role this recently described protein secretion mechanism plays in pathogenesis. Another exciting hypothesis is that proteins transported by EVs to the extracellular environment might achieve the same function inside or outside the cell. EVs might also have a role in intracellular communication as it has been reported for EVs of mammalian cells [78]. However, more data are needed to support these hypotheses.

Recently, Gil-Bona et al. and Wolf et al. presented evidence that mutants in *ECM33*, a gene that codifies for a GPI-anchored cell wall protein, and some lipid biosynthetic genes, *CHO1*, *PSD1* and *PSD2*, affect *C. albicans* EV cargo and morphology [66, 79]. Since there are a couple of theories about the biogenesis of the EVs in fungi from the invagination of the plasma membrane or the release of cytoplasmic compartments, and taking into account the fact that cell wall and plasma membrane proteins are components of *C. albicans* EVs, it is not surprising that mutants that have altered the cell wall and the phospholipid biosynthesis have exhibited differences in terms of the complexity and secretion of EV [55, 62]. These data support the theory that at least part of the EVs from *C. albicans* are formed from the cytoplasmic membrane, as reported for the mammalian microvesicles that are formed by the budding of the plasma membrane [80]. Furthermore, the proteomic composition of those vesicles that include a high number of plasma membrane proteins, such as the glucan synthase Gsc1 or Pma1 transmembrane proteins, and those involved in endocytosis and exocytosis also support this hypothesis. Indeed, the budding or remodelling of the plasma membrane as one of the mechanisms of the biogenesis of fungal EVs has already been proposed [62, 69, 76, 81]. The traffic of EVs carrying unconventionally secreted proteins from plasma membrane to the extracellular space in fungi prompts the question of how they are transported across the fungal cell wall, and although some hypotheses have been

proposed, further research is required to confirm them [69, 76, 81]. This is a very relevant point because, as described in the section above, the Eno1, Tdh3 and Hsp70 moonlighting proteins have been identified as secreted and as cell-wall-associated proteins for many years and EVs traffic can explain the presence of these unconventionally secreted proteins in both the extracellular medium and at the cell wall. More research that analyses separated *C. albicans* EVs and EV-free secretome of different mutants could help to clarify the mechanisms of the EVs biogenesis and their functions in the physiology of *C. albicans*.

5. Global view of extracellular proteins of *C. albicans*.

An integrated view of the data that has been produced by different proteomic works is presented in Table S1. This table shows all the proteins identified in at least two of the articles revised in this review, organised by extracellular localisation. More than 570 proteins are included representing the most extensive catalogue of *C. albicans* extracellular proteins to date. Sixteen of these proteins were identified in all the subproteomes analysed (plasma membrane, surfome, cell wall and secretome) and have been described in at least two different proteomic studies on surfome, cell wall and secretome (Table 1). Furthermore, 13 of them are immunogenic (marked with an asterisk in Table 1). This table shows that several classically cytoplasmic proteins such as Eft2, Eno1, Hsp70, Pdc1, Pgk1, Ssb1 or Tdh3 have been identified in all these locations [16]. Many of them are moonlighting proteins [16]. The detection of *C. albicans* Eno1 in the cell wall was described in depth in Section 2. Several approaches have also detected enolase in *S. cerevisiea* plasma membrane [43, 82]. Furthermore, some of these moonlighting proteins have been described as potential biomarkers for the diagnosis or prognosis of invasive candidiasis [5, 19, 20]. Table 1 also includes three GPI-anchored proteins: Ecm33, Pga4 and Phr2. Ecm33 is required for cell wall integrity, in cell wall regeneration, morphogenesis, chronological life span

and virulence [79, 83-85]. Pga4 is a β-glucan transglucosidase and Phr2 has a role in the proper cross-linking of beta-1,3- and beta-1,6-glucans [86, 87]. It is important to note that these three GPI-anchored proteins were also detected in the two analysed fractions of secretome (EVs and EV-free secretome). In conclusion, we were able to define a core of 13 proteins secreted by the classical or unconventional pathway that can be found in the cell membrane, cell wall and secretome, indicating their ubiquity and relevance in host-pathogen interaction.

The proteomic studies that were examined as part of this paper also demonstrate that *C. albicans* modifies their external protein profile to adapt to environmental or morphological changes as it adapts its cytoplasmic proteome; for example, as observed in the yeast-to-hypha transition [21, 27, 31, 37, 47, 88]. The cell surface protein composition changes between yeast and hyphal morphologies and also in biofilms. [36, 37]. Moreover, Table S1 also shows the proteins that were identified as secreted only by yeast or by hyphae cells.

6. Advantages and disadvantages of the different proteomic approaches

In this article, the proteomic approaches used to study *C. albicans* surface and extracellular proteins are revised focusing mainly on sample preparation and the results obtained. The proteomic technologies are not discussed in detail; however, it is important to highlight some relevant points. It is widely known that different approaches render complementary results. This has been demonstrated in studies that uses 2D-PAGE and LC-MS/MS approaches and within different LC-MS/MS approaches [34, 43, 47]. In this sense, 2D-PAGE exhibits some limitations to resolve very high or very low molecular weight proteins but is useful as a means of identifying different isoforms of the a protein that are resolved and visualised in different spots [39, 88, 90]. On the other hand, the LC-MS/MS approaches used in the works revised here could identify the high and low molecular proteins in a straightforward way but were unable to distinguish among

most isoforms. These LC-MS/MS approaches are bottom-up proteomic approaches that provides an indirect identification of proteins through peptides derived from proteolytic digestion [91, 92]. When this strategy is employed, the unmodified peptides from different protein isoforms are indistinguishable. Some modified peptides, such as the phosphorylated peptides can be identified, but these approaches are not useful in studies on more complex post-translational modifications such as glycosylation. Glycosylation is a relevant protein modification in many *C. albicans* cell wall and secreted proteins. This modification takes place as the proteins transit through the secretory pathway [5, 9, 10, 93]. In fact, the outer layer of the cell wall is comprised of glycosylated mannoproteins that are the first point of contact with the immune system. The relevance of glycosylation of *C. albicans* proteins in the immune recognition has been well known for years, for example for Mp65 [74, 93]. The 2D-PAGE based studies revised here are relevant for the study of the cell wall and secreted glycosylated proteins. Some highly glycosylated proteins, such as Hsp150 or Mp65, were detected as a smear at the top acidic part of the gel [21, 47]. These works also describe the use of serological proteome analysis (SERPA) to identify glycosylated or unglycosylated immunogenic proteins that can be relevant to the development of new diagnostic tests for candidiasis [21, 41, 47].

Nowadays, LC-MS/MS proteomics methods are very sensitive and fast; as such, they had been used for the analysis of the *C. albicans* surfome and secretome. These studies resulted in the generation of lists of hundreds of proteins and provide a global view of the complex protein composition of extracellular environments [37, 38, 47, 55]. This complexity is clearly shown in Table S1. The global vision of the proteomic composition of each of these cellular and extracellular locations is of relevance in efforts to understand the functions, processes or pathways that proteins are involved in and the key processes that take place in those locations, as

shown in this review. However, taking into account the high number of proteins in each analysis, the relative abundance of the proteins of each list or the most abundant proteins in each compartment are also of significance. It is important to note that, in all the subcellular fractionation processes minor contamination from a different subcellular location is possible.

The majority of the works that were commented in this review were more focused on protein identification than on relative quantification. Diverse gel and non-gel based approaches can been employed to study changes in the protein abundance in cells growing in different conditions [94]. As commented before, 2D-DIGE, that makes use of fluorescent dyes, was used for the study of non-covalently cell surface proteins from biofilm and planktonic cells and ^{15}N-metabolic labelling for the relative quantification of the dynamics covalently bound cell wall proteins in different growing conditions [25-31]. Different labelling techniques have not been used for the study of *C. albicans* extracellular proteins but the recent development of easy and robust label free LC-MS/MS approaches that are being widely used in proteomics will help the study of the dynamics of these proteins in more culture conditions pushing the biomarker discovery [95, 96].

7. Concluding remarks and perspectives.

This review highlights how the improvement of proteomic techniques and the use of the optimal approach has a considerably impact on the results obtained when analysing the external face of *C. albicans*. The most recent analysis employed cell shaving and identified hundreds of surface proteins including more than 30 GPI proteins in the cell surface of *C. albicans*, representing a significant increase on the number of proteins detected in previous studies. Several years ago, only 44 proteins had been identified in the secretome of *C. albicans* while the *in silico* prediction

identified more than 400 different proteins. Hundreds of proteins have now been identified in the secretome of *C. albicans* under different growth conditions. Furthermore, the number of immunogenic proteins described has also increased.

Taken together, the proteomic data that has been collected thus far provides a global overview of the dynamic surface of *C. albicans* and extracellular proteomes; mainly composed of secreted enzymes, including cell wall-related enzymes, hydrolases and adhesins (some of which are involved in pathogenesis) secreted through the classical secretory pathway, and metabolic proteins, stress proteins and elongation factors, some of which are moonlighting proteins, secreted by unconventional mechanisms. The protein composition and cell morphology of *C. albicans* changes in response to alterations in the environment thereby allowing the cells to adapt to different host niches; however, some abundant proteins are detected in several conditions. Thus, the extensive overview of the extracellular protein composition of *C. albicans* presented here could facilitate the advancement of new, faster, and more reliable diagnostic analysis and the development of effective treatments.

However, the current knowledge in this field remains limited in some aspects and more studies are required. Advances in proteomic techniques and further studies on specific proteins could answer the outstanding questions. The application of label-free proteomic approaches for the relative quantification of proteins could represent a useful approach through is possible to study the external face of *C. albicans.* This method coupled with high-precision MS will help researchers to develop a better overview of the proteome dynamics in the surface and secreted proteins of *C. albicans* during its different stages of growth. In addition, the incorporation of top-down proteomic approaches could represent a complementary approximation to the structural

analysis of extracellular proteins that is very useful for the identification of different forms of a single protein, including the analysis of post-translational modifications.

Finally, the discovery of EVs as an unconventional pathway of protein secretion provides an opportunity for the future therapeutic use of these compartments. However, there are still many unresolved questions related to this pathway. Currently, the knowledge of the protein composition of EVs of *C. albicans* is based on results of the study of yeasts in a limited number of growing conditions. The analysis of the EVs and EV-free secretome of different cell morphologies or growth under different conditions could explain if *C. albicans* uses these compartments to release specific proteins to the extracellular media, affecting its adaptation to the media, growth, nutrition or/and host-cell interactions. The answers may lie in future proteomic studies that focus on understanding the participation of these proteins in cell communication, as well host-pathogen interactions. In addition, the EVs pass through the cell wall to be relieved at extracellular medium by an unknown mechanism, consequently modifying the cell wall composition. The study of the mechanisms of EVs secretion could also generate new insights that could be use in the development of new therapeutic approaches.

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Figure legends

Figure 1: Schema outlining proteomic approaches for the study of the "external face" in *C. albicans*. According to sample preparation, on the left side approaches for the extraction of cell surface proteins (surfome) including plasma membrane and cell wall proteins are represented. The cell wall isolation allows the study of this fraction by protein extraction (cw1, cw2) or by trypsin shaving of isolated cell walls (cw3). To study the global surfome, the approaches use whole cells that can be analysed by extraction of proteins (sf1) or directly by trypsinization of live cells in the "cell shaving approach" (sf2). On the right side, strategies for the study of secreted proteins as whole secretome (sc1 and sc2) or after separation of EVs and EVs-free secretome (sc3) are represented. After sample preparation proteins can be separated by gel-base strategies or analysed by MS-based approaches. For further details and references see the text.

Figure 2: Venn diagrams of the common proteins found in *C. albicans* extracellular vesicles from different strains. a) Thee clinical isolates grown at the same temperature 30ºC but in different conditions: SC5314 strain was cultivated for 16h in SD medium [54] and strains 11 and ATCC 90028 were cultivated for 48h in Sabouraud medium [64]; b) SC5314 strain was cultivated at different temperatures and growing conditions: 30 ºC for 16h in SD medium [64] or 37 °C for 72 h in YPD medium [65]. c) The four conditions tested.

Table 1. Proteins identified in *C.albicans* cell membrane, cell wall, surface and secretome. The table includes proteins identified in at least two proteomic studies of cell wall, cell surface and secretome referenced in text (in brackets). Proteins indicated with an asterisk are immunogenic [19, 38, 41] (unpublished data for Ecm33).

Table 1

Standard name	Systematic name	Membrane	Cell wall			Surfome		Whole secretome			Evs	Vesicle free secretome
		Yeast	Yeast	Hypha	Mix yeast+ hypha	Yeast	Hypha	Yeast	Hypha	Mix yeast + hypha	Yeast	Yeast
Adh1*	C5_05050W_A	[43]	[21, 22]	[21]		[14, 31, 35, 36, 37]	[31, 36, 37, 38]		[47]		[66]	
Bmh1	C1_03220C_A	[43]	[21, 22]	[21]		[14, 36, 37]	[36, 37, 38]		[47]		[66]	
Cdc19*	C2_05460W_A	[43]	[21, 22]	[21]		[14, 36, 37]	[36, 37, 38]		[47]		[66]	
Cdc48	C1_10790W_A	[43]	[21, 22]	[21]		[36, 37]	[36, 37, 38]	[47]	[47]		[66]	
Ecm33*	C1_03190C_A	[43]	[22, 23, 24, 26, 27, 28]	[27, 28, 29]	[25, 30]	[36, 37]	[37, 38]	[15, 26, 47]	[28, 47]	[15, 25]	[55, 66]	[55]
Eft2*	C2_03100W_A	[43]	[21, 22]	[21]		[14, 35, 36, 37]	[36, 37, 38]		[47]		[55, 65, 66]	
Eno1*	C1_08500C_A	[43]	[21, 22]	[21]		[31, 35, 36, 37]	[31, 36, 37, 38]	[15, 47]	[15, 47]	[15, 25]	[55, 65, 66]	
Fba1*	C4_01750C_A	[43]	[21, 22]	[21]		[31, 35, 36, 37]	[31, 36, 37, 38]		[15, 47]	[25]	[65, 66]	
Hsp70*	C1_13480W_A	[43]	[21, 22]	[21]		[14, 31, 36, 37]	[14, 31, 36, 37, 38]		[47]		[55, 65, 66]	
Hsp90*	C7_02030W_A	[43]	[21, 22]	[21]		[35, 36, 37]	[14, 36, 37, 38]	[47]	[47]	[15, 25]	[66]	
Pdc11*	C4_06570C_A	[43]	[21, 22]	[21]		[31, 35, 36, 37]	[14, 31, 36, 37, 38]		[47]		[55, 65, 66]	
Pga4*	C5_05390C_A	[43]	[22, 23, 24, 26, 27, 28]	[27, 28, 29]	[25, 30]	[35, 36, 37]	[37, 38]	[15, 26, 47]	[28, 47]	[15, 25]	[55]	[55]
Pgk1*	C6_00750C_A	[43]	[21, 22]	[21]		[31, 35, 36, 37]	[14, 31, 36, 37, 38]		[15, 47]		[55, 65, 66]	
Phr2	C1_00220W_A	[43]	[22, 23, 26, 27, 28]	[27, 28, 29]	[25, 30]	[35, 36, 37]	[37, 38]	[26, 47]		[25]	[55, 65, 66]	[55]
Ssb1*	CR_08090W_A	[43]	[21, 22]	[21]		[14, 31, 35, 36, 37]	[31, 36, 37, 38]		[47]		[65, 66]	
Tdh3*	C3_06870W_A	[43]	[21, 22]	[21]		[14, 35, 36, 37]	[14, 36, 37, 38]	[47]	[47]	[15, 25]	[55, 65, 66]	

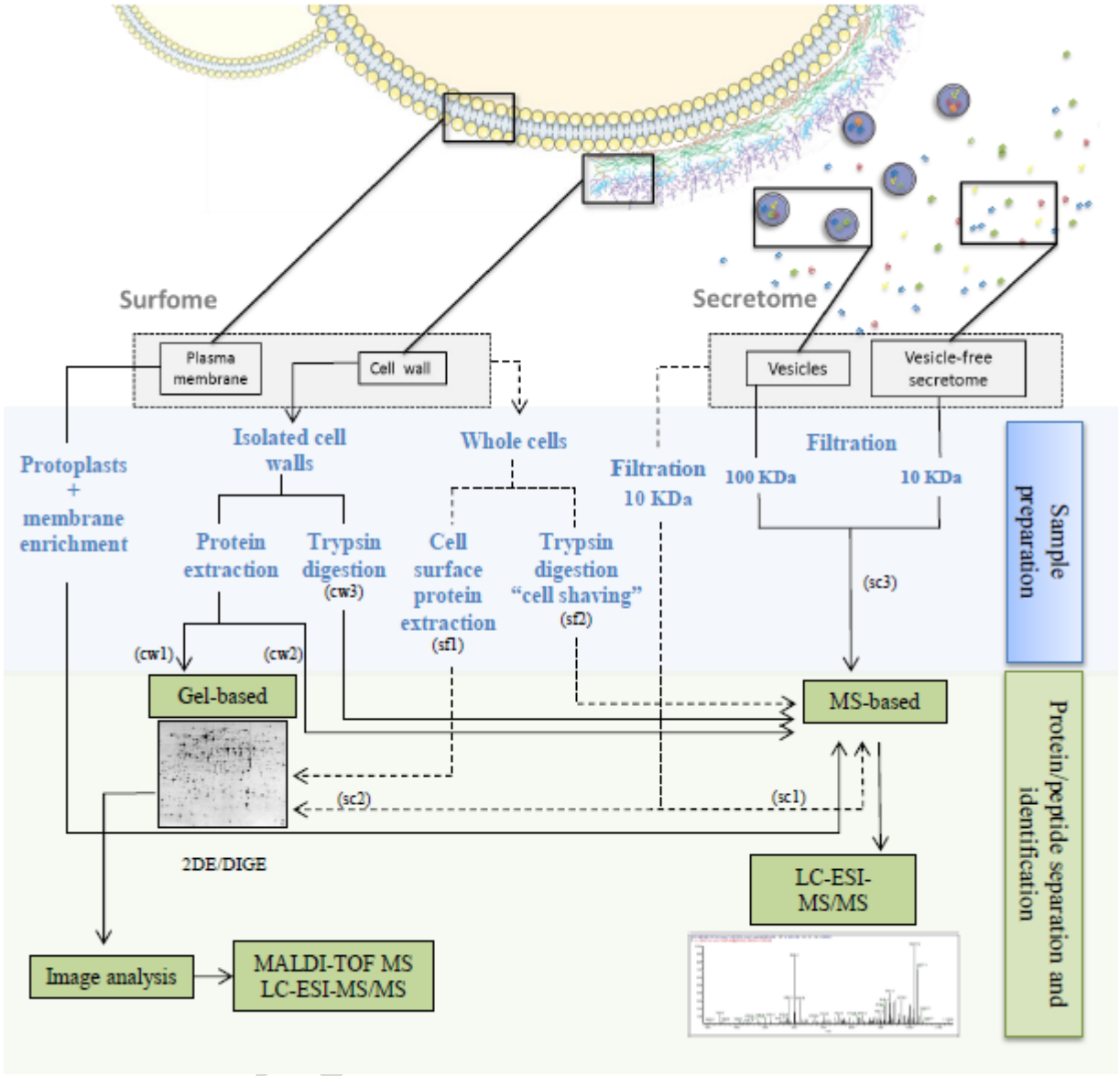


Figure 1

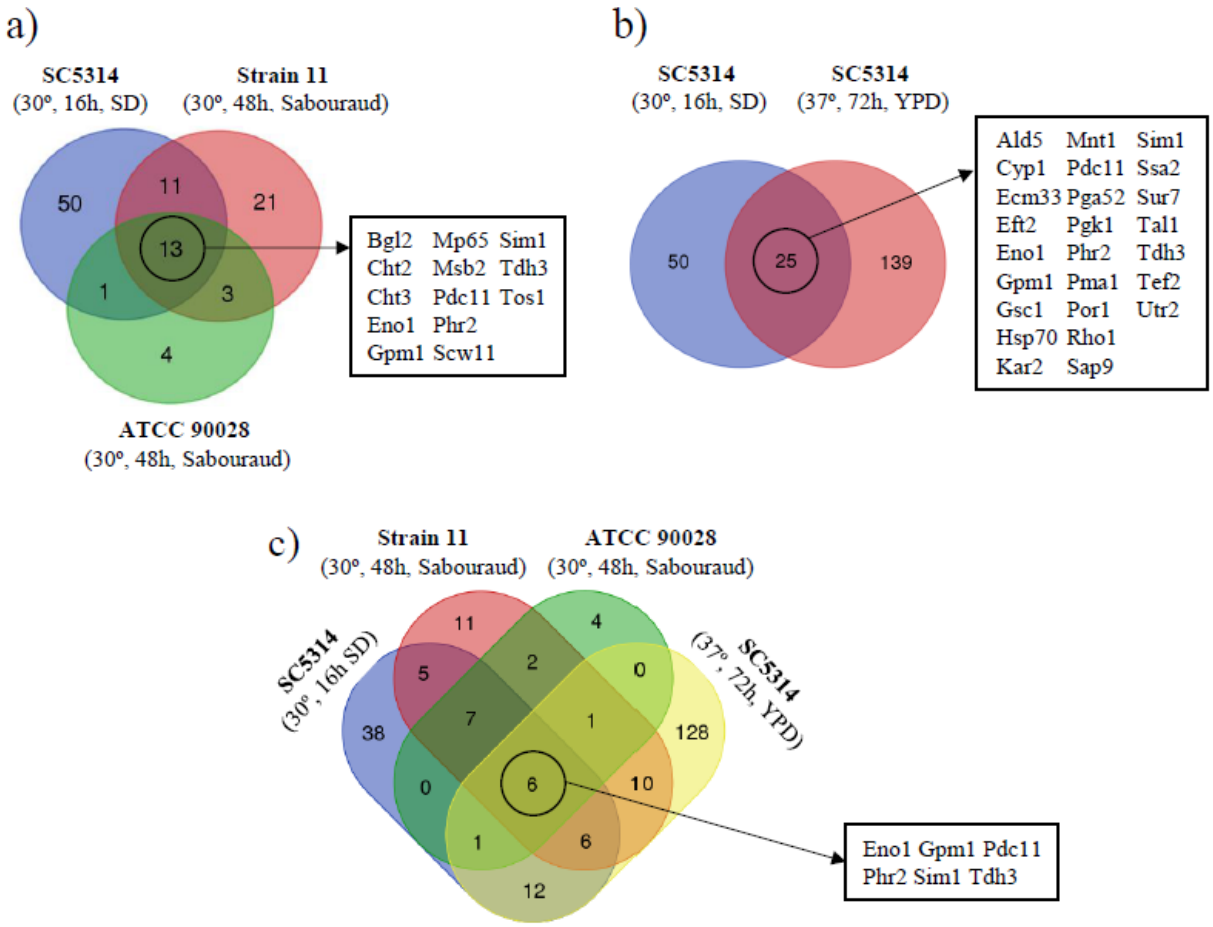
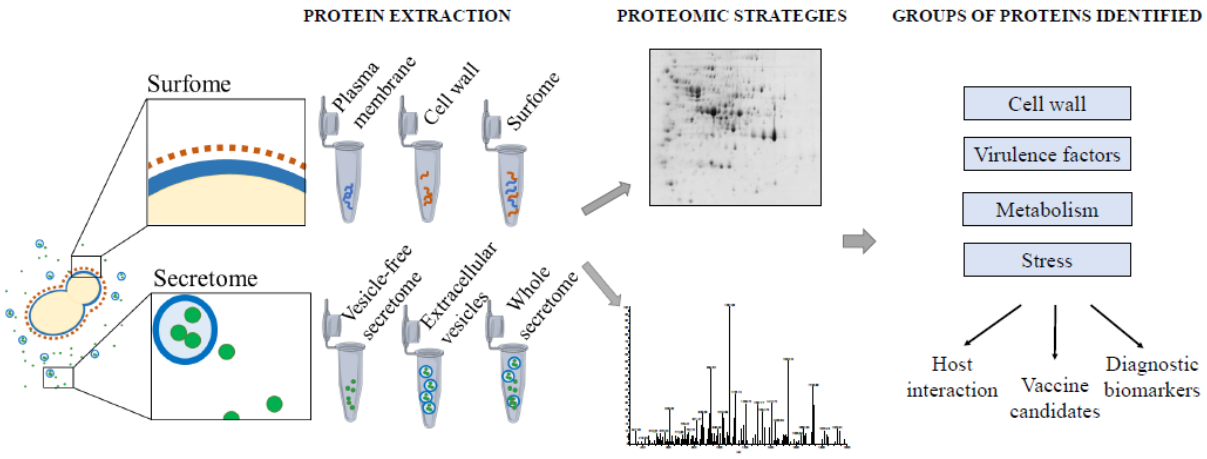


Figure 2



Graphical Abstract

Highlights

1. Global view of the proteins in *C. albicans* surfome and secretome.
2. Review of proteomic approaches used to obtain and analyse these subproteomes.
3. Extracellular vesicles as an unconventional secretory pathway in *C. albicans.*
4. Set of common proteins in the cytoplasmic membrane, the cell wall and the secretome.