Mass spectrometric quantification of Candida albicans surface proteins to identify new diagnostic markers and targets for vaccine development

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Scope

Throughout this thesis, we have examined the importance of proteins of the opportunistic fungal pathogen *Candida albicans* that are either secreted into the environment or covalently anchored to the cell wall. Using and further developing a mass spectrometric approach first introduced by de Groot and Yin *et al.* for *C. albicans* [1,2], we were able to relatively quantify wall proteins and laid the groundwork for absolute quantification of these proteins. Furthermore, we identified proteins in the medium that are critical for the remodeling of the cell wall both during growth and under stress conditions. Additionally, as the conditions that are required for the expression of specific proteins differ significantly, we hypothesized how the discrepancy between the predicted number of secreted proteins can be reconciled with the lower number of detected proteins. In line with this, we discussed the importance of protein families with members of different specificity or conditional activity. To put the proteins we identified in context with their function, we extensively reviewed literature and attempted to develop new hypotheses in how proteins of the cell surface interact with each other and the host. We also discussed how *C. albicans* is able to evade or mitigate the attack of the immune system. Taken together, we identified promising proteins for marker and vaccine development in the wall proteome and the secretome. The research done in the course of my thesis is summarized in the following 15 concepts.

1. Proteomics and transcriptomics supplement each other

Proteomics is often considered secondary to transcriptomic analysis, confirming previously obtained expression results. Nonetheless, large discrepancies between the two methods are regularly observed [3]. Proteomic quantification is an endpoint measurement, showing the final concentration of the protein at its point of destination and often is also informative to the extent and character of post-translational modifications. On the other hand, transcriptomic quantification occurs at the level of the mRNA after transcription. While mRNA levels are often indicative of the concentration of intracellular, non-targeted proteins, they are less accurate for proteins undergoing trafficking and modification after translation. In addition, the number of transcript generated per time can vary considerably and differential mRNA stability (*e.g.* through polyA length) also affects the amount of protein generated from one mRNA before its decay [4].
The translated protein levels might also differ due to protein turnover, degradation and misfolding [5]. Then the protein is post-translationally modified, which again introduces room for errors compared to the mRNA levels. For secreted proteins, the modified protein is then going through the secretory pathway, where accidental or deliberate retention can occur leading to reduced protein amounts at the point of destination. For wall-anchored GPI proteins, there is also the potential for washing out the precursor before attachment. Therefore, a combinatorial approach of transcriptomics and quantitative proteomics is desirable.

2. Secretome size and lifestyle are linked

As already discussed in chapter 4, the proportion of predicted secreted proteins compared to the entire proteome varies considerably between approx. 2.1% for example in *Pichia pastoris* and *Kluyveromyces lactis* to approx. 7.4% proteins in *Aspergillus nidulans*. Many pathogenic fungi including *Candida albicans* (~3.1%), fall between these two extremes with approx. 2.3-3.4% [6]. The saprophytic fungi from, for example, the *Aspergillus* genus, have by far the most secreted proteins with a diverse complement of enzymes involved in the degradation of dead plant and animal material [7]. Some species are pathogenic, but infections are accidental, since the fungi are soil-dwelling and do not generally co-exist within a human host. Therefore, immune evasion is not a primary evolutionary pressure. Similarly, non-pathogenic yeast are usually adapted to very specific conditions with respect to carbon source and pH optima, which might result in a “streamlined” secretome specifically geared towards these conditions at the cost of reduced adaptability. In contrast, *C. albicans* and many other pathogenic fungi regularly colonize human mucosa with high prevalence, leading to predation by the immune system to keep them in check without causing severe disease, as is often observed in immune-compromised patients. This might have led to “streamlining” of the secretome, to avoid immune detection by reducing the number of secreted proteins in general compared to saprophytes as well as stringent control of expression based on environmental conditions.

Unlike the non-pathogenic yeasts, pathogenic fungi also produce a set of secreted proteins directly related to infection, *e.g.* the Sap proteins of *C. albicans* that are involved in nutrient acquisition and immune evasion. This added set increases secretome size but the expression is also tightly controlled. Of the pathogens in the *Candida* genus, *Candida albicans* is the most successful with the
largest predicted secretome of 166 soluble proteins with signal peptides. This might be related to the fact that other pathogenic Candida species do not readily form true hyphae, but only pseudo-hyphae. As we have seen in chapter 5, hyphal growth leads to the expression of specific hypha-associated proteins that play an important role for example in adhesion to host tissue and the formation of biofilms. Overall, it seems there is a strong correlation of secretome size with lifestyle, with opportunistic pathogenic fungi taking the middle ground of coexistence with the immune-competent host, only causing disease when the immune status is reduced, while saprophytic fungi directly attack host tissue upon infection leading to a strong immune response and non-pathogenic yeasts lack the essential tools to regularly facilitate effective infections in immune-competent humans.

3. Protein families allow optimal functionality under diverse conditions

C. albicans is able to thrive in many host niches, including the skin, mucosal surfaces, the blood stream, and internal organs. Wall proteins are subject to the surrounding conditions and come into contact with highly diverse, niche-associated, extracellular matrix proteins from the host as well as with bacterial surface proteins. This probably explains the evolution of many wall protein families with individual members showing optimal functionality dependent on environmental conditions and infection sites [8]. Interestingly, invasive growth is generally associated with hyphal growth and comparison of the wall proteomes of yeast and hyphal cells revealed a core set of hypha-associated wall proteins under various hyphal growth-inducing conditions (Als3, Hwp1, Hwp2, Hyr1, Plb5, and Sod5) [9,10].

The two largest wall protein families are the Als family [11] and the Hyr/Iff family [12]. The family of ALS (agglutinin-like sequence) proteins consists of eight GPI-modified, elongated, broad-specificity adhesins with an immunoglobulin-like N-terminal domain that can interact with a wide variety of host proteins [13]. Some Als proteins possess amyloid-forming sequences, which could play a role in forming biofilms [14]. Fascinatingly, Als3 has multiple functions, including ferritin binding [15] as well as binding to E-cadherin, thereby facilitating iron uptake and active internalization of C. albicans by host cells, respectively [16]. This supports that proteins of a family may share a particular function, but might also have additional functions, which are not conserved throughout the family. Intriguingly, Hyr1, one of the twelve GPI-
proteins belonging to the Iff/Hyr family, is strongly hypha-associated and con-
ers resistance to neutrophil killing [17] through its N-terminal domain. Alt-
ough the domain structure within the family is variable, the N-terminal domain
is strongly conserved in all family members (Figure 7.3A) [12]. This hints at a
more general, niche-specific role of the family in evading immune cells under
der different growth conditions.

The environmental pH also strongly affects the wall proteome, revealing the
preferred usage of specific family members at acidic and neutral pH [18]. The
same is true for protein families in the secretome, which has been shown best in
the family of the secreted aspartyl proteases (Figure 7.1). Borg-von Zepelin and
coworkers showed that Sap1-3 activity is highest at pH 3-5, while the activity
of the hyphal-specific Saps 4-6 is optimal at pH 5-7 [19]. In this context it is
important to note that vaginal mucosa has an acidic pH, while the oral cavity
has a near neutral pH, further cementing the niche specificity of Saps. Intrin-
seingly, the two covalently anchored members of the Sap family, Sap9 and
Sap10, have a much broader pH range from pH 5-8 [20]. Functionally, both
seem to be more important for the remodeling of the cell wall and its proteins
instead of specifically targeting host tissue.

Figure 7.1. Dendrogram of the phylogenetic relationship of the Sap family (from
[21]).

Sap1-3 are associated with low pH and the yeast form, and Sap4-6 are mainly associ-
ed with hyphae and growth at near neutral pH. Sap7 and Sap8 are opaque-associated,
Sap7 is also associated with oral infections and Sap8 with vaginal infections. Sap9/10
are GPI-anchored yapsin-like members of the Sap family and play an important role in
cell wall maintenance and the processing of wall proteins and have high similarity with
the yapsin Yps2/Mkc7 of *S. cerevisiae*. 
4. Morphological changes lead to the expression of specific wall proteins
The yeast-to-hypha transition is the major morphological transition in the life cycle of *C. albicans* and also plays a major role in virulence. Hyphae are able to penetrate physical barriers, *e.g.* host tissues and cells, and are also important to establish persisting biofilms. Hyphal formation can be induced by different means, most successfully by fetal calf serum (FCS), as well as its most active substituent muramyl dipeptides (MDPs) [22], by certain amino acids like proline [23] and by *N*-acetylglucosamine (GlcNAc) [24]. Intriguingly, although the induction methods and the pathways involved are different, the response with regard to the expression of wall proteins is highly similar (Figure 7.3C; chapter 5). While a set of proteins, most notably wall remodeling enzymes, are not strongly affected by the morphological change, the added set of proteins expressed in hyphae is important for successful infection. This is supported by the much smaller incidence of infections by *C. dubliniensis*, a very closely related sister species of *C. albicans*, which only rarely forms true hyphae. Wall proteins produced by hyphae assist in adhesion (Hwp1, Als3), coping with oxidative stress (Sod5) and seem to play a role in immunevasion (Hyr1). As already noted before, the secreted aspartyl proteases Sap4-6 are also strongly associated with the hyphal morphology and play an important role in host tissue destruction, for example to acquire nutrients. This suggests that proteins that are hypha-specific are an added set of proteins that are not essential, but lead to increased pathogenicity and infection success.

5. Nutrient acquisition from host cells requires the secretome
Many proteins of the secretome are hydrolytic enzymes that attack host tissue and cells leading to the release of nutrients. Members of the lipase (Lip), phospholipase (Plb), glucoamylase (Gca), and Sap families are secreted into the environment, tightly regulated depending on the environmental conditions. There they breakdown proteins and carbohydrates that can then be easily taken up by *C. albicans* (Figure 7.2). For example, Sap2 is able to degrade collagen, laminin, fibronectin and mucin, among other substrates [25]. In addition, Saps also play an important role in immune evasion by degrading host defence proteins (discussed later) [26]. The importance of Sap secretion for virulence has been shown in all infection models, including the oral cavity and the vaginal mucosa [25].
While the role of proteases for infection and nutrient acquisition is clearly shown, the role of lipases, phospholipases and other hydrolyzing enzymes is not as clear. Only group B phospholipases have so far been detected in the secretome of \textit{C. albicans} (chapter 3). Since they are able to hydrolyze components of the cell membrane, they probably play a role in the acquisition of nutrients from host tissue, vesicles or dead cells. A knockout mutant of plb1 shows reduced virulence in a murine model and decreased gastric tissue penetration [27]. Similarly, lipases are important for lipid utilization at an oil/water interface, but their expression is not causally linked to the presence of lipids [28]. Lastly, \( \beta-N\)-acetylhexosaminidase, encoded by \textit{HEX1}, is involved in the utilization of \( \text{N-} \text{acetylglucosamine (GlcNAc), abundantly present on host glycoproteins, as carbon and nitrogen source [29], but its optimal activity at pH 4 [30] might explain why we did not detect it in the secretome in the presence of GlcNAc, since we used pH 7.4-buffered medium (chapter 3). Overall, the entire repertoire of hydrolyzing enzymes in the secretome assists in acquiring nutrients with high specificity for the surrounding conditions. This again underlines the importance of protein families.

6. Trace metal acquisition in the human body involves surface proteins

One of the most restricted nutrients in the human body is iron. Because of its reactive nature but also in order to restrict growth of invading microorganisms, free iron is highly limited in the host and mainly found in association with proteins, either as a prosthetic group like in hemoglobin and myoglobin, stored inside ferritin, transported by transferrin, or liganded by lactoferrin. \textit{C. albicans} has evolved a number of strategies to scavenge iron from these complexes (Figure 7.3B). As mentioned above, Als3 is also important for iron acquisition as a receptor for ferritin, an iron-storage host macromolecule that contains about 30\% of the total human iron pool. Without Als3 \textit{C. albicans} is unable to grow on ferritin as sole iron source [15]. Additionally, the five Rbt5 family proteins, characterized by an internal domain containing eight invariantly spaced cysteines [31] also play an important role in iron acquisition. Csa1, Pga7, Pga10, and Rbt5 are found attached both to the plasma membrane and the wall, while Csa2 is secreted [32-35]. It has been shown that Csa1, Pga10, and Rbt5 are involved in heme binding [36]. As the expression of \textit{CSA1, PGA7, PGA10, and RBT5} is co-regulated under various conditions including iron restriction, the question arises whether the Rbt5 family proteins might act as a relay system, similar to
bacterial iron uptake systems [37]. Similarly to Csa2, Pra1 is a secreted protein that has been shown to be involved in zinc acquisition [38]. The “zincophore” Pra1 is released into the host cell plasma after hyphal penetration (Figure 7.2). There it scavenges free or complex-associated zinc and then re-associates with the fungus, followed by uptake through zinc transporters [38]. These specific iron and zinc acquisition methods, which are facilitated by wall proteins and members of the secretome, underline the importance of trace metals during infections.

7. Wall remodeling is carried out by carbohydrate-active enzymes

Cell shape is mainly determined by the skeletal polysaccharides of the wall, which are important for resisting the internal turgor pressure and shielding the cell from external mechanical forces. Nonetheless, remodeling of the wall is required, for example, during isotropic growth and cell separation, formation of new buds and hyphal branches, and for coping with surface stress. Remodeling of the wall is mediated by wall- and plasma membrane-resident, carbohydrate-active enzymes that detach, re-arrange and re-attach carbohydrates. This remodeling is also evident by the regular presence of GPI-proteins in the medium (chapters 3, 4 and 6). The main wall-bound proteins involved are a chitinase (Cht2), transglucosylases (Phr1, Phr2, Pga4) and chitin transglycosylases (Crh11, Utr2). The secretory aspartyl proteases Sap9 and 10, and Pir1, a predicted β-glucan cross-linking protein, also seem to be involved in wall remodeling and maintaining wall integrity [21,32]. In contrast to Sap1 to 8, Sap9 and 10 are GPI-modified, yapsin-like proteases that are retained at the cell surface [21]. Interestingly, Sap9 has been implicated in the processing and shedding of other wall proteins, most notably, the chitinase Cht2 and Pir1 [20]. The levels of wall-bound Sap9 seem largely morphotype-independent, but its levels increase in conjunction with surface stress conditions as observed in response to fluconazole [32].

In addition to the wall-bound carbohydrate-active enzymes, secretome proteins also play an important role. The secreted chitinase Cht3, the endoglucanase Eng1 and the glucanase Scw11 play an important role in cell separation, while the β-transglucosylase Bgl2 and the exo-glucanase Xog1 together with the wall-bound Phr1 are contributing to the delivery of carbohydrate material to the extracellular matrix [39]. Mp65, Sim1, Sun42, both containing a SUN domain and contributing to the cell wall stability of the mother cell after separation, and
Tos1 are also involved in wall remodeling, but their actual roles and activities are less clear.

**Figure 7.2. Important functions of secreted proteins during *C. albicans* growth and infection.**

Bgl2 and Xog1 deliver carbohydrates to the extracellular matrix (ECM). Mp65, Sim1 and Sun41 contribute to a stable mother cell wall, while Cht3, Eng1 and Scw11 are expressed in the daughter cell and lead to proper cell separation. The signaling mucin Msb2 is processed by surface-resident proteases and the large extracellular domain is released into the environment, where it can bind to antimicrobial peptides (AMPs) of the host while the intracellular domain activates Mkc1 signaling. Lipases (Lips), Phospholipases (Plbs) and Secreted aspartyl proteases (Saps) are released into the medium and attack host tissue. Upon hyphal penetration of a cell, soluble Pra1 is released into the host cytoplasm and scavenges zinc. Zinc-loaded Pra1 then re-associates with the hypha and is taken up.
8. Cell separation requires proteins from the secretome

Especially during cell separation, carbohydrate-active enzymes play an important role in severing the carbohydrate connections between mother and daughter cell. Before final separation, the cytoplasm of both cells is already separated but the cells are still connected by the wall, mainly through the primary septum, which consists of chitin, but also the peripheral wall in the neck region. To achieve full separation, these connecting structures need to be dissolved by the activity of chitinases and glucanases (Figure 7.2). The expression of the endoglucanase Eng1 and the chitinase Cht3 is daughter cell-specific and regulated by the transcription factor Ace2 [40]. Knockout mutants of \textit{CHT3} as well as \textit{ACE2} lead to a strong non-separation phenotype, while the \textit{ENG1} knockout phenotype is less pronounced (chapter 6). Interestingly, while cell separation is impaired by reduced chitinase levels, the stress resistance of the cells increases. This is most likely directly related to increased chitin levels in the wall, which are associated with higher resistance to stress [41]. Chitin levels increase either as a result of increased chitin synthesis [42,43] or through decreased chitin degradation (chapter 6), but most likely through a combination of both. This suggests that there are two competing drives within the cell, cell separation and increased stress resistance. Apparently, stress resistance is favored evolutionary over cell separation, because the abundance of chitinases is decreased in the presence of a stressor. At the site of cell separation, the cell wall and the membrane are already slightly stressed and any additional stress would be detrimental.

9. How do the wall and its proteins cope with surface stress?

Surface stress resistance is an important property during infection. \textit{C. albicans} is exposed to many different stresses during infection, including the action of antifungals like fluconazole, affecting the membrane, and caspofungin, directly affecting the wall. Furthermore, we could show that any kind of membrane stress also results in changes in the cell wall [32] (chapter 6). But also other factors like the presence of mixed carbon sources and temperature affect the cell wall and its proteins. Strikingly, when \textit{C. albicans} is grown on a poor carbon source such as lactate (found in the vaginal fluid and together with acetate maintaining its acidic pH [18]), or on a mixture of lactate and glucose, the cell wall gets significantly thinner and more flexible. Importantly, these alterations
are accompanied by substantial changes in the wall proteome [44]. Through this and other similar studies we have identified a core set (Crh11, Phr1, Phr2, Pga4, Sap9, Utr2) of wall-remodeling proteins that is conserved in the response to several surface-stress conditions [32,44] (chapter 6). Conceivably, this protein set could also be important to survive other surface stresses, including membrane-perturbing antimicrobial peptides found in body fluids, epithelial layers, and immune cells. The functional domains of these proteins are conserved in the Ascomycotina, suggesting similar importance for other fungi as well. The increased resistance of *C. albicans* to surface stress can be most likely attributed to an increase in this core set together with the reinforcement of the cell wall with chitin as described before.

**Figure 7.3. Key concepts of the wall proteome of *C. albicans.*
Center: TEM picture of the cell wall and its proteins (courtesy of Iuliana V. Ene and Alistair J.P. Brown, Aberdeen). A. Domain structure of the Hyr/Iff family (adapted from [12]). From left to right: N-terminal signal peptide; white box: conserved domain; dark grey box: Ser/Thr-rich region; light grey box: Asp/Gly-rich region; black box: GPI-anchor addition signal. B. Iron acquisition through wall proteins. Rbt5 family proteins bind hemoglobin, while Als3 is the receptor for ferritin. Iron from ferritin and
transferrin is sequestered via the reductive iron uptake system. C. Effect of yeast-to-hypha transition on the wall proteome with yeast-associated (open squares; upper panel), morphotype-independent (grey squares) and hypha-associated (closed squares, lower panel) proteins [9]. D. Interaction of wall proteins with the immune system. Wall-resident superoxide dismutases (Sods) detoxify reactive oxygen species (ROS) [45]. Hyr/Iff family proteins confer resistance to immune cell killing [17]. Cell wall proteases process Msb2 and liberate the extracellular domain Msb2*. Msb2* binds to antimicrobial peptides (AMPs) in a dose-dependent manner and confers resistance [46].

10. Adhesins facilitate the formation of biofilms

Stress resistance of *C. albicans* and especially antifungal resistance also increase through the formation of a biofilm. A biofilm is a surface-associated community of micro-organisms of either the same species or a mixture of species encased in an extracellular matrix [47]. They often occur in catheters and other in-dwelling or implanted devices and pose a serious threat to patient health. They are established stepwise, first by adherence of yeast cells to the surface, which is followed by formation of hyphae and extracellular matrix and maturation of the biofilm (Figure 7.4) [47]. Throughout the establishment of a biofilm, wall proteins and especially the adhesins of the Als family play an important role. The hypha-associated proteins Als3 and Hwp1 most likely provide robustness to the biofilm through adhesion to both the surface and surrounding cells [48,49]. Als2 seems to stabilize the mature biofilm in a similar fashion [50,51]. Furthermore, it seems that the aforementioned transcription factor Ace2, which regulates many cell wall targets and is important for cell separation, is also involved in biofilm formation [52]. Impressive work by Nobile and coworkers over the years established that biofilm formation is regulated by an intricate network of six transcription factors (Bcr1, Brg1, Efg1, Ndt80, Rob1, Tec1), of which many regulate the expression of wall proteins and proteins of the secretome [49,53-56]. This includes hypha-associated proteins (Als3, Ece1, Hwp1, Hyr1), transglycosylases (Cht2, Phr1) and adhesins (Als1-3). This underlines the importance of the surface proteome in facilitating adherence to the substrate and neighboring cells as well as in providing extracellular matrix.
11. The role of the secretome in matrix and biofilm formation

The extracellular matrix (ECM) plays an important role in formation of biofilms and contributes to resistance by sequestering antifungal compounds, *e.g.* fluconazole [57]. Sequestration leads to an order of magnitude higher resistance to fluconazole, even without the acquisition of specific mutations resulting in higher drug resistance [58-62]. The extracellular matrix is a mixture of extracellular polymers, with β-1,3-glucan being the major carbohydrate constituent, nucleic acids, and some proteins (Figure 7.5). The soluble β-1,3-glucan of the ECM is released from the insoluble glucan of the cell wall. Zap1 is a transcription factor that regulates gene expression based on the zinc status of the cell. Carbohydrate delivery to the matrix is either Zap1-repressed [63] or Zap1-independent [39], with both pathways affecting secretome proteins. Zap1 represses the expression of the glucoamylases Gca1 and Gca2 in the presence of zinc, in contrast to the situation for Pra1, suggesting reduced production of ECM material during zinc starvation. As we already saw in this chapter and earlier in chapter 4, zinc is severely limited in the human body, suggesting that extracellular matrix is only produced in conditions where trace metals are available and a biofilm is established to remain near the zinc source. Gca1 and Gca2 release β-1,3-glucan from the cell wall [63]. Similarly, but Zap1-independent, the transglucosylase Bgl2 together with the exo-glucanase Xog1 and the GPI-anchored transglucosylase Phr1 form a matrix glucan delivery system [39].
12. Immune evasion is mediated by wall proteins

*C. albicans* has evolved various mechanisms to avoid or counteract the immune response (Figure 7.3D). The cell wall is the first line of defense, but also a target for the immune system due to its recognition sites for the innate immune system and its immunogenic epitopes. For example, the receptor dectin-1, which is mainly expressed on dendritic cells and macrophages, recognizes the β-glucan of the wall and leads to the activation of pro-inflammatory cytokines [64]. However, the mannoprotein coat largely prevents the detection of the underlying β-glucan layer. Additionally, the wall protein Hyr1 effectively reduces immune cell killing of *C. albicans* [17]. In support of its protective role, heterologous expression of Hyr1 in *Candida glabrata* also mitigates immune cell killing, suggesting a direct function of the protein. *C. albicans* also has two wall-bound, morphotype-associated superoxide dismutases (Sod4, Sod5) [33]. These cell wall-resident Sods are essential for dealing with extracellular ROS (reactive oxygen species), resulting from the oxidative burst - a general mechanism of immune cells to kill invading pathogens. As expected, *SOD4* and *SOD5* knockout mutants are more susceptible to oxidative stress [45]. Sod6, another GPI-anchored member of the Sod family, has not been detected in proteomic screens and gene deletion did not reveal a clear phenotype [45]. Furthermore, Saps are also able to degrade human complement, a set of host defense proteins [26]. Antimicrobial peptides, like histatins, defensins, and cathelicidins, belong to the
arsenal of host defense mechanisms as well. Recently, the shedding of the extracellular part of the plasma membrane-bound signaling mucin Msb2 (Figures 7.2 and 7.3D), which is involved in maintaining cell wall integrity, has been shown to convey resistance to histatin-5 and the cathelicidin LL-37 in a dose-dependent manner [46]. This processing and shedding is not mediated by Sap9/10 but most likely other surface-resident proteases, since the \( S. \) \textit{cerevisiae} homolog ScMsb2 is processed by the Sap9 homolog Yps1 [65].

An interesting conundrum is the efficacy of glycolytic enzymes like enolase or fructose-bisphosphate aldolase as vaccines and targets for the immune system. From our current point of view, although these enzymes are not readily exposed on the surface, where they can be detected by immune cells, it is conceivable that they are second line targets for the immune system. While the surface proteins of \( C. \) \textit{albicans} are recognized first by the immune system and have evolved to be as little immunogenic as possible, the intracellular proteins are more immunogenic. One could imagine that after the initial interaction of immune cells and fungal cells, these highly immunogenic targets become available from lysing cells and result in additional immune cells being recruited to the site of infection. This could lead to a faster clearance of the infection and therefore improve outcome and survival.

13. Core set proteins could improve diagnostics by serving as biomarkers

Earlier diagnosis of life-threatening infections is key to increased survival of patients. This is complicated by fast clearance of fungal proteins in the bloodstream by the kidneys, as well as the often severely reduced immune status of patients, leading to very low antibody titers. Therefore, screening of patient serum for a single protein or antibody directed against a fungal target is often either too late or not specific enough for proper treatment. Interestingly, Mp65, a member of the secretome core set (chapter 3), has already been used to a certain extent as a biomarker [66]. By using the combined secretome and wall proteome core sets (sections 7 and 9) as combined biomarkers, it is conceivable to develop a protein- or antibody-based ELISA directed against all proteins of these sets. An increased number of targets in the assay, would lead to increased specificity for \( C. \) \textit{albicans} identification in patient serum. This is important for assigning the right treatment option, \textit{e.g.} fluconazole, since other \textit{Candida} species have a much higher resistance to antifungals. Furthermore, a highly specific single target ELISA could still miss low abundant proteins or antibodies in se-
rums. Multiplexing a diagnostic assay based on core set proteins would reduce the number of false positives while increasing the performance of the assay even in the low concentration range most likely encountered in the serum of immune-compromised patients.

14. Which wall proteins are promising targets for vaccine development?
A vaccine that could be administered to risk groups, e.g. pre-surgery, or to women suffering from recurrent vaginitis, would be an important asset. As stated earlier, the functional domain of wall proteins is almost exclusively situated in the N-terminal region, while the C-terminal part is mainly of structural importance. This is reflected in the various vaccines that are currently being developed (reviewed in [8,67]). For example, mice immunized with the recombinantly expressed N-terminal domain of Als3 become resistant to infections by *C. albicans* as well as *Staphylococcus aureus* [68]. The N-terminal domains of Als1 and Hyr1, and a short immunogenic peptide from the N-terminal domain of Hwp1 conjugated to a β-1,2-linked mannotrioside, have been used similarly as *C. albicans* vaccines [8,17]. Notably, these four vaccine targets are strongly associated with hyphae, suggesting that hyphal epitopes might be more easily recognized by the immune system as a threat, since they are associated with the breaching of host tissue. Invasive growth *in vivo* is not only associated with hyphal growth, but probably also with iron restriction and thus with increased levels of the Rbt5 family of iron acquisition proteins in the wall [34]. Relevantly, all five members of the this family contain an identical sequence (with Csa1 containing four copies) that could represent a prime target. Developing this approach further, it is conceivable to combine immunogenic epitopes from the N-terminal functional region of a selection of wall proteins in a single recombinant protein for use as a multi-component vaccine, similar to the QconCAT approach described in chapter 2. The power of this method is twofold, firstly, the modular design of the QconCAT allows matching and mixing of epitopes based on experimental results, leading to an optimal vaccine protein. Additionally, this approach is more robust since even when parts of the QconCAT vaccine fail or lead to adverse side effects, they can be easily replaced. Secondly, using epitopes for multiple targets leads to a highly effective molecular “shotgun burst” to the targeted cell by the immune system, which is also more difficult to evade.
15. Outlook and future research opportunities

In summary, the evolution of wall protein families in the human fungal pathogen *C. albicans* allows survival in diverse host niches and has resulted in an impressive plasticity of the wall proteome. The exposure of wall proteins on the surface and proteins of the secretome in the medium together with their critical functions, makes them promising targets to combat fungal infections. Furthermore, the use of antibody- or protein-based diagnostic assays and single- or multi-component vaccines has the potential to improve patient outcome significantly. While the concepts developed and discussed in this thesis are excellent starting points for development of new therapeutic and diagnostic methods, their practicality and efficiency remains to be tested.

The experimental approach for the identification and quantification of wall proteins and the secretome are solid and useful. Nonetheless, absolute quantification using AQUA or QconCAT methods would multiply the returns with regard to both fundamental research and applied, clinical solutions. In addition, the advantages of combining imaging techniques and mass spectrometric analysis for surface and wall proteins is self-evident. In closing, as discussed throughout this thesis, continued basic and applied research, application of cutting-edge methods and better understanding of the concepts of *C. albicans* infections will lead to faster diagnostics, better treatment options resulting in less suffering and more lives saved.
References


