Diagnosis of invasive candidiasis: From gold standard methods to promising leading-edge technologies

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Abbreviations

CAGTA	*Candida albicans* germ tube antibody
ELISA	enzyme-linked immunosorbent assay
ESCMID	European Society of Clinical Microbiology and Infectious Diseases
FDA	Food and Drug Administration
IC	invasive candidiasis
ITS	internal transcribed spacer
MALDI-TOF	matrix-assisted laser desorption/ionization time-of-flight
MS	mass spectrometry
PCR	polymerase chain reaction
PNA-FISH	peptide nucleic acid-fluorescent in situ hybridization
T2RM	T2 magnetic resonance

Abstract

Invasive candidiasis (IC) poses a major public health problem worldwide. Despite the introduction of new antifungal agents and changes in clinical practices, its morbidity and mortality rates and healthcare costs remain persistently high. This is mainly because of the serious underlying conditions of infected patients (critically ill or severely immunocompromised patients) and the difficulties encountered in early diagnosing this opportunistic mycosis and initiating prompt and appropriate antifungal therapy. In the light of this great clinical challenge, the past decades have witnessed the development of diverse early detection and therapeutic intervention strategies aimed at minimizing the clinical impact and economic burden of this healthcare-associated infection caused by *Candida* species. Here, we review the currently available methods for IC diagnosis. These encompass (*i*) gold standard methods (fungal culture and tissue histopathology), (*ii*) pathogen-derived biomarker detection tests (PCR, protein antigens, mannan, ß-D-glucan and D-arabinitol-based assays), (*iii*) host-derived biomarker detection tests (*Candida albicans* germ tube antibodies or CAGTA, anti-mannan antibodies, other infection-specific antibodies, procalcitonin, serum amyloid A, interleukin 17, interleukin 23 and transforming growth factor ß-based assays), (*iv*) clinical prediction algorithms (*Candida* score, colonization index and other prediction rules), and (*v*) leading-edge molecular, proteomic and immunomic technologies (such as peptide nucleic acid-fluorescent in situ hybridization or PNA-FISH, T2 magnetic resonance or T2Candida assay, and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry or MALDI-TOF MS, among others). Their strengths, utility, limitations as well as combined use to assist in the diagnosis of this life-threatening and costly fungal infection (including candidemia and deep-seated candidiasis) are also discussed.

1. Introduction

Invasive candidiasis (IC) is the most common fungal infection acquired in healthcare-associated settings (particularly in intensive care, surgery, transplant, hematology-oncology, burn and neonate units) [1-3]. This nosocomial infection is directly linked to advances in medical care directed towards prolonging patient survival [2, 4]. These include important risk factors for IC, such as the wider use of broad-spectrum antibiotics, more aggressive immunosuppressive treatments (new chemotherapeutic, cytotoxic or immunomodulatory agents, and solid organ or hematopoietic stem cell transplantation), invasive medical devices (central vascular catheters and ventilators), total parenteral nutrition, hemodialysis, major surgical procedures and intensive care, to name but a few [2, 5-7]. Although this healthcare-associated fungal infection can be acquired exogenously through the hands of hospital workers or contaminated solutions, most cases of IC are endogenous in origin and stem from the patient microbiota and opportunistic nature of its etiological agents [8-9].

This opportunistic mycosis is caused by several *Candida* species. These are common members of the normal microbiota on the oropharyngeal cavity, gastrointestinal tract and vagina of many healthy individuals [10-12]. However, these harmless commensal fungi can take advantage of an opportunity not normally available, induce key virulence factors, and cause invasive infection [4, 13]. The chance that allows them to evolve into harmful opportunistic pathogens is closely related to alterations in the normal microbiota composition (by the repeated or long-term use of broad-spectrum antibiotics), disruptions in the natural cutaneous and gastrointestinal barriers (by invasive procedures, burns or wounds), and impairments of the immune system (by disease o immunosuppressive therapies) [4, 7, 14-15].

Invasive forms of *Candida* infection encompass candidemia (bloodstream infection) and deep-seated candidiasis (infection involving other normally sterile body fluids and tissues) [1, 4,

16]. Candidemia is often associated with a sepsis syndrome (similar to severe bacterial infections) and deep-seated infections in different organs (such as eye, kidney, liver, spleen, brain, bone, abdominal cavity, heart and lung) [4, 14, 17].

2. Epidemiology of IC

2.1. Causative species

In most clinical settings, *Candida albicans* is the most prevalent etiologic agent of IC [4, 8]. Nevertheless, other non-*albicans Candida* species, such as *Candida glabrata, Candida parapsilosis*, *Candida tropicalis*, *Candida krusei* (currently known as *Pichia kudriavzevii* [18]) and *Candida auris*, have recently emerged as major causative species in some high-risk populations due to changes in clinical practices [4, 19-21]. More specifically, this shift towards other non-*albicans Candida* species appears to be the result of the selective pressure associated with the prophylactic or preemptive use of first-line antifungals that have worked very effectively against invasive infections caused by *C. albicans*, such azoles (in particular, fluconazole) and echinocandins [17, 22]. For instance, the emergence of *C. glabrata* and *C. parapsilosis* is associated with settings with the heavy use of fluconazole and echinocandins, respectively, as these non-*albicans Candida* species are less sensitive to such antifungal agents than *C. albicans* [4, 17]. In view of the different drug susceptibilities of *Candida* spp., accurate species-specific identification is essential for choosing proper antifungal therapy as well as reducing selection pressure for antifungal resistance among clinical isolates [1, 4].

The distribution of *Candida* spp. varies considerably over time and with geographical location, institution, hospital ward, pre-exposure to antifungal agents, underlying disease or age, among other factors [8, 20, 22-23]. For example, *C. glabrata* is commonly isolated in Northern

Europe, the United States and Canada, while *C. parapsilosis* has emerged as a major cause of IC in Southern Europe, South America and Asia [17, 19, 21, 24-25]. In addition, *C. glabrata* and *C. parapsilosis* are most prominently isolated in the elderly and neonates, respectively [8, 22, 24].

The morphological forms observed in infected tissues from IC patients also differ among *Candida* species (Figure 1) [26]. *C. glabrata* only grows as small yeasts (ovoid-shaped, budding, unicellular cells), whereas *C. parapsilosis*, *C. krusei/P. kudriavzevii* and *C. auris* are dimorphic fungi capable of exhibiting both yeast forms and pseudohyphal forms (multicellular filaments with constrictions at their septa) in clinical lesions [26-27]. In contrast, *C. albicans* and *C. tropicalis* are polymorphic or pleomorphic fungi that can form yeasts, pseudohyphae and true hyphae (multicellular filaments with parallel walls at their septa) in infected tissues and promote different stages of the infectious process [13, 15, 26, 28-29].

2.2. Incidence

The incidence of IC also varies substantially over time and depending on several factors, such as geographic location, local epidemiology, institution, hospital ward, infecting *Candida* spp., underlying condition or age, to name but a few [4, 8, 24]. In particular, its incidence rates are more elevated among critically ill and severely immunocompromised patients as well as among patients at the extremes of age [8, 17, 24-25, 30]. Diverse population-based surveillance studies have revealed annual incidence rates of candidemia of 2-14 cases per 100 000 persons, which were higher in the United States than in Europe [8, 24-25, 31-33]. Remarkably, *Candida* spp. were the most common etiologic agents of primary bloodstream infections and the seventh leading cause of all healthcare-associated infections, corresponding to 22% and 6%, respectively, of all cases in the United States in 2011 [34].

2.3. Impact on public health

Unfortunately, IC remains a relentless public health problem worldwide that affects over 250 000 people every year and accounts for over 50 000 deaths on the basis of conservative estimates [8, 17]. This healthcare-associated fungal infection adds significantly to the morbidity and mortality of hospitalized patients, especially those with severe underlying conditions (Figure 2) [8, 17, 35]. Mortality rates among IC patients also differ over time and with infecting *Candida* species, clinical management, institution, hospital ward, underlying diseases or age, among others [8, 30, 36-40]. These ranged from 31% to 47% in several epidemiologic and clinical studies conducted in different Spanish hospitals [24, 41-42]. Similar trends have also been highlighted in the United States and other European countries [1, 8, 25, 35, 43-46]. This infectious disease is also responsible for extensive healthcare costs (US $6 214-142 394 per patient), which come from longer hospitalizations (ranging between one and several weeks) and more expensive antifungal treatments [8, 17, 38-39, 47]. In addition to these elevated hospital costs, IC also contributes to substantial societal costs (like lost productivity) [8]. The elevated mortality rates, length of hospital stay, as well as hospital and societal costs attributable to IC could considerably be minimized by timely initiation of antifungal therapy [8, 24, 36-37]. Each day of delayed treatment increases mortality risk around 50% and is associated with an additional US $5 000 in healthcare costs [8, 37, 48].

3. Diagnosis of IC

Early detection is therefore crucial for reducing the impact on patient clinical outcome and economic burden of this life-threatening and costly mycosis [8, 24, 36-37]. However, IC is extremely difficult to diagnose at an early stage because of its unspecific clinical signs and

symptoms (except for *Candida* endophthalmitis and chorioretinitis), and the insufficient accuracy and time delay of the currently available diagnostic methods [4, 8, 14, 30, 49-51] (Figure 2). As a consequence, the diagnosis of IC is often reached in advanced stages of infection (where antifungal therapy is most likely to fail and patient clinical outcome is poor) or undesirably at autopsy [4, 16, 37, 52].

The next sections will provide an overview of the currently available tools for the diagnosis of IC. These mainly include the current gold standards, nonculture laboratory methods based on measurement of pathogen and host-derived biomarkers, clinical prediction scores and highly promising leading-edge technologies (Figure 3). Their advantages and disadvantages, as well as combined use, will be also discussed.

4. The gold standard methods for IC diagnosis

Fungal culture and tissue histopathology from normally sterile sites remain the gold standards to diagnose IC [16]. Owing to their high specificity, a positive result of these conventional techniques allows the clinicians to firmly establish a definitive diagnosis of IC and thus initiate the administration of antifungal therapy [4, 50]. However, these direct detection methods of invasive fungal infection have major drawbacks (Figure 4), which would in part account for the persistently elevated morbidity and mortality rates attributable to this opportunistic mycosis [8, 37].

4.1. Fungal culture

Blood cultures are still the gold standard method for the diagnosis of candidemia (the most obvious manifestation of IC) in the absence of or associated with deep-seated candidiasis.

On the contrary, these are useless for the detection of deep-seated candidiasis in the absence of candidemia [16, 53]. Overall, their sensitivity is too low (21-71%) in the first stages of infection and decreases in the presence of nonviable *Candida* cells [16]. In fact, these can remain negative in the presence of antifungal drugs that had been administrated as prophylactic or preemptive treatment before blood specimen collection [16, 54-55]. On the other hand, blood cultures can take around 2-5 days to achieve conclusive results. More specifically, these require a median time to positivity (for fungal growth) from 19 hours for *C. tropicalis* to 75 hours for *C. glabrata*, and another 24-48 hours for subsequent species-specific identification using conventional methods, such as Vitek® 2 YST ID card (bioMérieuxe), AuxaColor™ 2 (Bio-Rad Laboratories), or CHROMagar™ Candida (CHROMagar), among others [16, 56-58]. For instance, CHROMagar™ Candida is a differential, chromogenic solid medium that allows the presumptive identification of clinically important *Candida* species (Figure 1) [26, 59]. Lamentably, this slow turnaround time leads to delayed therapeutic interventions with ensuing poor outcomes of IC patients [37].

Fungal cultures from tissue and sterile body fluids (other than blood) collected from deep sites of infection are considered the gold standard methods to diagnose deep-seated candidiasis in the absence of candidemia [4, 16]. Similar to blood cultures, these are also relatively insensitive and time-consuming laboratory techniques [4, 16, 50]. In addition, the invasive procedures applied in their sampling are commonly inappropriate or even unfeasible in most patients at risk for IC as argued below [53].

4.2. Tissue histopathology

Histopathological detection of *Candida* spp. on biopsy specimens from normally sterile sites enables the confirmation of invasive disease as well as an assessment of tissue invasion and inflammation [4, 50]. Nevertheless, this conventional method may become particularly invasive

and risky in unstable or critically ill patients, or even contraindicated in patients undergoing severe thrombocytopenia or coagulopathies [14, 53]. Like fungal cultures, its sensitivity is also reduced. This may be attributed to low burdens or uneven distributions of viable *Candida* cells in infected tissues as well as the difficulties encountered in identifying optimal tissue biopsy sampling sites [16, 50, 53]. Furthermore, its diagnostic usefulness may also be limited in patients who have received prophylactic or preemptive therapy prior to deep tissue biopsy sampling [30].

5. Nonculture laboratory methods for IC diagnosis based on measurement of pathogen-derived biomarkers

In an attempt to overcome the main limitations of fungal culture and tissue histopathology (Figure 4), diverse alternative laboratory methods (*i.e.* nonculture-based and minimally invasive assays with a quick turnaround time) have been developed and assessed for rapid IC diagnosis over the past few decades. These are based on measurement and evaluation of biomarkers derived either from the pathogen (*Candida* nucleic acids, proteins and carbohydrates) or from the host (human proteins) in clinical specimens (such as blood, serum, plasma or urine, among other samples) from patients at risk for IC (Figure 5) [49, 60-61]. As defined by the Biomarkers Definitions Working Group, a biomarker or biological marker is "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" [62].

Although several of these indirect detection methods of invasive fungal infection are commercially available, most of them remain investigational (Figure 5). However, none of them has shown to be sufficiently accurate or reliable to attain widespread clinical use and improve the therapeutic decision-making process on its own as detailed below. This section will survey those

based on measurement of *Candida* biomarkers for early detection of this severe and costly fungal infection, while the next section will outline those based on human biomarkers.

5.1. Candida nucleic acids

5.1.1. Candida DNA

Many commercial and in-house polymerase chain reaction (PCR)-based assays have been developed and evaluated for detection of *Candida* DNA in diverse biospecimens (such as blood, serum, plasma, cerebrospinal fluid and tissues) from patients at risk for IC [50]. These have employed a wide variety of DNA targets to diagnose IC, which range from *Candida*-specific genes (such as *ERG11*, *HSP90*, *SAP1-6*, *CHS1* or *ACT1*, among others) to highly-conserved multi-copy broad-range panfungal genes (such as 5.8S, 18S or 28S ribosomal sequences or the internal transcribed spacer (ITS) 1 or ITS2 regions within the rRNA gene) [1, 14, 63]. In addition to these fungus-specific assays, there are also several commercial broader multiplex PCR-based assays targeting bacterial and fungal pathogens (including different *Candida* spp.). These multiplex platforms have been applied directly to clinical specimens, such as LightCycler® SeptiFast (Roche Diagnostics), SepsiTest™ (Molzym), Magicplex™ system (Seegene), or VYOO® (SIRS-Lab), and to positive blood culture bottles, like BioFire FilmArray® (bioMérieux), for IC detection [50, 55, 64].

Despite their considerably high pooled sensitivity and specificity for IC diagnosis reported in a meta-analysis of 54 studies [63], these PCR-based assays have important drawbacks. On the one hand, these have not yet been methodologically standardized for routine clinical practice nor have they been validated in large multicenter clinical trials, thereby hampering their widespread implementation [17, 49]. On the other hand, these assays can yield false-negative results because of low numbers of fungal cells in blood as well as complications

during sample preparation (*e.g.*, inefficacious cell breakage and DNA extraction due to the presence of the fungal cell wall) [32, 39-40, 53]. Likewise, these are also prone to false-positive results on account of similarity between fungal and human DNA as well as exogenous sample contamination (with DNA from ubiquitous fungal pathogens in buffers, in the environment, on utensils and on skin) [4].

5.2. Candida proteins

At present, there is only a commercially available assay for measurement and evaluation of *Candida* protein biomarkers in patients at risk for IC. This is a latex agglutination test based on the detection of uncharacterized, heat-labile *Candida* protein antigens in serum (CAND-TEC™; Ramco Laboratories, Inc.). Unfortunately, this assay has proven to be low sensitive for IC identification [65]. Although several biochemically-defined *Candida* protein antigens (such as a 47-kDa fragment of Hsp90, Eno1, Mp65 or Sap1/2, to name but a few) have also been identified as potential IC biomarkers in serum, overall antigen detection assays have limited diagnostic usefulness for IC [49, 66-71]. Specifically, the detection of *Candida* protein antigens is seriously hindered by their rapid clearance from the bloodstream, formation of immune complexes with their related antibodies as well as their low concentrations in the circulation [49]. These limitations may, to some extent, be solved by the use of serial determinations for these antigen detection assays [70].

5.3. Candida carbohydrates

5.3.1. Candida cell wall polysaccharides

The *Candida* cell wall is the outermost cellular structure, and is composed of (*i*) polysaccharides, including mannans (32-36%) found solely in covalent association with

proteins, ß-D-glucans (47-60%) formed by (1,3)-ß and (1,6)-ß linkages, and chitin (0.6-9%), (*ii*) proteins (6-25%), and (*iii*) lipids (1-7%) [15, 72]. This basically consists of an elastic three-dimensional framework of microfibrillar polysaccharides (ß-D-glucans and chitin) that surrounds the plasma membrane and to which mannoproteins and proteins are anchored mostly on its outside in different ways (Figure 6A) [72-77]. Its two most abundant constituents (mannan and (1,3)-ß-D-glucan) are important polysaccharide biomarkers for IC diagnosis, and can be detected by several commercial assays as specified below [1, 4].

5.3.1.1. Candida mannan

Candida mannan can be measured and evaluated by a latex agglutination test (Pastorex® *Candida*; Bio-Rad Laboratories) or a sandwich enzyme-linked immunosorbent assay (ELISA) (Platelia™ *Candida* Ag Plus; Bio-Rad Laboratories) in serum or plasma from patients at risk for IC. Similar to protein antigen detection assays, these have also proven to have low sensitivity to diagnose IC [65, 78]. This is mainly because of the high immunogenicity of mannan, which is rapidly cleared from the circulation and may also form immune complexes with circulating anti-mannan antibodies, thereby complicating their assessment [49]. The reduced potential of these assays for IC diagnosis can be enhanced by the combined detection of *Candida* mannan antigen (mannanemia) and human anti-mannan antibodies in serum or plasma (see the next section) [79].

5.3.1.2. Candida (1,3)-ß-D-glucan

Several commercial assays have also been developed for the detection of circulating *Candida* (1,3)-ß-D-glucan in serum or blood from patients at risk for IC, such as Fungitell® (Associates of Cape Cod, Inc.) and Fungitec G-MK® (Seikagaku). These are based on the ability of (1,3)-ß-D-glucan to activate a horseshoe crab proteolytic coagulation cascade [80-81]. The

Fungitell® assay was approved by the United States Food and Drug Administration (FDA) in 2004 to assist in IC diagnosis, and remains the most used test in daily clinical practice in Europe and the United States [82]. The current European Society of Clinical Microbiology and Infectious Diseases (ESCMID) guidelines recommend its use in the diagnosis of candidemia and other forms of IC in adults (Figure 6B), and suggest the use of serial determinations to improve its clinical performance and rule invasive *Candida* infection out [82-83].

Nevertheless these assays have some limitations. Because (1,3)-ß-D-glucan (a major cell wall component of many medically important fungi) is a panfungal biomarker of invasive fungal infection, this does not discriminate between infections caused by *Candida* spp. and other fungal pathogens, and require further species-specific identification [1, 4, 80]. Furthermore, these assays can lead to false-positive results in high-risk populations due to many potential sources of contamination. These include human blood products (coagulation factors, immunoglobulins, albumin and plasma protein fractions), hemodialysis, high triglycerides, surgical gauzes or other glucan-containing materials, excess sample manipulation, certain antibiotics (*e.g.* intravenous amoxicillin-clavulanic acid or piperacillin-tazobactam), some systemic bacterial infections and severe mucositis, to name but a few [4, 16, 80, 84-85].

5.3.2. Candida polyalcohols

5.3.2.1. Candida D-arabinitol

D-arabinitol, a metabolite produced by several clinically relevant *Candida* spp. (except for *C. glabrata* and *C. krusei/P. kudriavzevi*), has been measured and assessed in serum or urine from patients at risk for IC using different techniques [49, 86-88]. These range from gas chromatography methods to enzymatic-colorimetric/fluorimetric assays (based on quantification of NADH production or NADH-dependent end products) [49, 88].

These assays are prone to false-positive results because of the presence of endogenous L-arabinitol and D-arabinitol (optical isomers or enantiomers) in human serum and urine as well as increased serum levels of arabinitol (cleared from the bloodstream by glomerular filtration) in patients with renal dysfunction (Figure 7) [49, 88]. The effects of these confounding factors can be adjusted by calculating (*i*) the ratio of D-arabinitol to L-arabinitol (no fungal origin) concentrations [89-91], as well as (*ii*) the ratio of D-arabinitol to creatinine (an indicator of renal dysfunction) concentrations [87].

On the other hand, these assays do not provide species-level identification for *Candida*, and can also yield false-negative results when D-arabinitol non-producer *Candida* species are the only etiological agents of IC [87-88]. Furthermore, none of these D-arabinitol quantification assays has proven to be sufficiently sensitive and specific to diagnose IC on its own [87, 89, 91] nor have they been standardized methodologically and validated in large multicenter clinical studies.

6. Nonculture laboratory methods for IC diagnosis based on measurement of host-derived biomarkers

As an alternative option to solve the problems associated with conventional diagnostic methods for IC (insensitive, time-consuming and invasive techniques; Figure 4), several commercial and in-house nonculture laboratory assays based on measurement of host-derived biomarkers have also been developed and evaluated in patients at risk for IC over the past decades (see above). These biomarkers mainly encompass human antibodies directed against *Candida* protein and carbohydrate antigens, as well as other human proteins related to the host response against infection (Figure 5) [4, 61, 92]. Unfortunately, none of these assays has shown

to have the diagnostic accuracy necessary to attain widespread implementation on its own as discussed below.

6.1. Human antibodies

Antibody detection assays can lead to false-positive results in healthy individuals (because some *Candida* species are commensal members of human microbiota) as well as false-negative results in immunocompromised patients (who may be unable to mount a strong antibody response to *Candida* infection) [49]. These disadvantages may to a certain extent be circumvented by the use of (*i*) more sensitive antibody detection techniques, (*ii*) infection-specific antigenic protein species or antigenic epitopes rather than crude antigens as immunodiagnostic reagents, (*iii*) combinations of several antibody biomarkers, and (*iv*) combined antigen-antibody detection assays, among others [93-99].

6.1.1. Human antibodies to Candida protein antigens

There are two commercial *C. albicans* germ tube antibody (CAGTA) assays for IC diagnosis, which are based on an indirect immunofluorescence test [IC (CAGTA) IFA IgG, Vircell Microbiologists] and an indirect chemiluminescent immunoassay [IC (CAGTA) VirClia®, Vircell Microbiologists]. Both assays detect IgG antibodies to *C. albicans* cell surface antigens from germ tubes. These CAGTA tests have shown high specificity but moderate sensitivity for IC identification [100-101]. Nonetheless, several studies have revealed that their combined use with (1,3)-ß-D-glucan or mannan detection assays may enhance their diagnostic accuracy [102-104].

Diverse in-house immunoassays have also been developed for evaluating serum antibodies directed against one or several recombinant *C. albicans* protein antigens rather than crude antigenic protein extracts in patients at risk for IC [67, 94, 98, 105-110]. Despite their

potential to assist in IC diagnosis, these assays have not yet been validated in multicenter prospective cohort studies.

6.1.2. Human antibodies to Candida carbohydrate antigens

Circulating anti-mannan antibodies from patients at risk for IC can be measured with a commercial indirect ELISA (Platelia™ *Candida* Ab Plus; Bio-Rad Laboratories). Similar to anti-*Candida* protein antibody detection assays, this test has also limited utility for IC diagnosis [79]. As aforementioned, the diagnostic accuracy of this biomarker can be improved when this indirect ELISA is used in association with its corresponding sandwich ELISA for measurement of *Candida* mannan (Platelia™ *Candida* Ag Plus) in serum or plasma from patients at risk for IC (Figure 8) [79, 91, 99, 111]. The current ESCMID guidelines recommend the combined use of these mannan antigen and anti-mannan antibody quantification assays as well as serial determinations for both assays in the diagnosis of candidemia and chronic disseminated candidiasis but not for other forms of IC (Figure 6B) [82].

Because of the potential of IgG2 antibodies against *Candida* phosphopeptidomannan to discriminate IC and heavily colonized patients from non-infected, non-colonized or lightly colonized patients, this biomarker could aid clinicians in the initiation of early preemptive therapy [112]. However, their diagnostic accuracy has not been compared to the other IgG subclass antibodies. In fact, the clinical utility of IgG1, IgG2, IgG3 and IgG4 antibodies to *Candida* proteins and carbohydrates in patients at risk for IC remains to be evaluated.

6.2. Other human proteins

The diagnostic performance of several human proteins involved in the immune response against infection (other than antibodies) and inflammation has also been investigated in patients

at risk for IC [61, 65, 103, 113-115]. In particular, procalcitonin (PCT), serum amyloid A (SAA), interleukin 17 (IL17), interleukin 23 (IL23) and transforming growth factor ß (TGF-ß), among other acute phase proteins and pro-inflammatory cytokines, have been reported as potential diagnostic biomarkers for IC [61, 103, 113, 115]. However, these host-derived biomarker candidates have not yet proven to be sufficiently accurate to reach widespread clinical use on their own, nor have they been validated in large multicenter clinical studies [61, 65, 103]. Although their combined use with pathogen-derived biomarkers could enhance their diagnostic accuracy for IC, there are apparently contradictory results with the combination of procalcitonin with ß-D-glucan testing [65, 114].

7. Clinical prediction algorithms for IC diagnosis

In the light of the shortcomings associated with the current gold standards and nonculture laboratory methods mentioned above, diverse clinical prediction scores, indexes or rules based on the combination of multiple independent risk factors for IC have also been devised with the intention of identifying patients at risk for IC who may benefit from early therapeutic interventions [8, 14, 116]. These include the *Candida* score, *Candida* colonization index, peritonitis score, candidemia risk score, and other clinical prediction rules [116-124]. For example, the *Candida* score consists of a linear combination of four independent IC risk factors (multifocal *Candida* species colonization, surgery on intensive care unit admission, severe sepsis and total parenteral nutrition) with different statistical weights [117]. A prospective multicenter study highlighted an irrelevant IC incidence rate in non-neutropenic adult patients that had a *Candida* score of less than 3, had been admitted to the intensive care unit for at least 7 days, and had not received antifungal therapy [125].

In most settings, negative predictive values of these clinical prediction algorithms are high, but their positive predictive values are relatively low [8, 30, 116]. Although these tools may therefore be useful for ruling IC out and stopping administration of unnecessary prophylactic or empirical antifungal therapy, their potential for ruling IC in is reduced. However, their diagnostic accuracy may be improved by their combination with IC biomarkers, such as (1,3)-ß-D-glucan, CAGTA or procalcitonin, among others, and assessment in at least two consecutive samples (Figure 9) [113, 126-127].

On the other hand, the scoring systems that draw on colonization data (such as the *Candida* score and colonization index) are work-intensive and expensive, and have limited bedside practicability [30, 116]. In addition, their cost-effectiveness for the clinical management of IC remains unknown.

8. Leading-edge technologies for IC diagnosis

The past decades have witnessed an increase in the number of cutting-edge technologies introduced not only in research but also in the clinical mycology laboratory with the overriding intention of reaching a faster and more accurate diagnosis of IC. These are based on molecular, proteomic or immunomic approaches. Some of these promising leading-edge technologies are commercially available and cleared by the FDA as an aid for IC diagnosis (Figure 10).

8.1. Molecular methods

Several innovative molecular assays for the detection of *Candida* species from positive blood culture bottles are commercially available. These comprise the Yeast Traffic Light® PNA-FISH® assay (AdvanDx) and Prove-it™ Sepsis StripArray (Mobidiag) based on a broad-range

PCR-coupled microarray (PCR/microarray), to name but a few [64, 128-131]. In particular, the Yeast Traffic Light® PNA-FISH® assay is a fluorescent *in situ* hybridization (FISH) method approved by the FDA for IC diagnosis (Figure 10) that utilizes peptide nucleic acid (PNA) probes to target *Candida*-specific rRNA (26S rRNA). This assay allows the rapid and accurate detection of five medically important *Candida* species (*C. albicans, C. glabrata, C. parapsilosis, C. tropicalis* and *C. krusei/P. kudriavzevii*) directly from positive blood culture bottles [130]. Species-specific identification time is therefore reduced from 24-48 hours (using conventional methods; see above) to 30-90 min (using this molecular diagnostic method). One of the main disadvantages of the PNA-FISH assay is its reliance on positive blood cultures (which can be negative in up to 50% of IC cases, and need 19-75 hours for fungal growth [16, 49, 56, 58]). This assay may also yield false-negative results when other *Candida* species are the only causative agents of IC.

In order to solve the problem of dependence on positive blood cultures, leading-edge nonculture molecular diagnostic assays have alternatively been developed and evaluated for a rapid identification of *Candida* species directly from clinical samples. These include the T2Candida® assay (T2Biosystems) and a broad-range PCR-coupled electrospray ionization mass spectrometry platform (PCR/ESI-MS), among others [54-55, 132-133]. The T2Candida® assay is a miniaturized molecular method recently cleared by the FDA for rapid diagnosis of IC (Figure 10) that combines PCR with T2 magnetic resonance (T2MR). This assay enables the detection of the amplified DNA (hybridized to iron-decorated nanoparticles) from five clinically relevant *Candida* species (*C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis* and *C. krusei/P. kudriavzevii*) at concentrations of 1-3 colony-forming units per milliliter (CFUs/mL) in whole-blood specimens within 3-4 hours [54, 134]. This new nanodiagnostic method has shown to be highly sensitive and specific to diagnose candidemia [54, 133-135], and does not

need viable *Candida* cells or sample purification and preparation (unlike blood cultures or other biosensors, respectively) [48, 54, 133]. According to economic and outcome modeling studies, the T2Candida® assay has the potential to save hospital costs (US $26 887 per patient with candidemia) as well as reduce candidemia-related mortality (60.6%) [56, 136]. However, this promising molecular diagnostic method has also some shortcomings. This assay relies on the prevalence of IC in each clinical setting, and needs further validation in cases of candidemia caused by other *Candida* spp. and deep-seated candidiasis (especially in the absence of candidemia) [134]. Furthermore, its costs are elevated (US $350 per sample), the expiration date of its reagents is very short, and its sensitivity may decrease in the absence of intact *Candida* cells in whole-blood samples [54, 56].

8.2. Proteomic methods

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has recently emerged as a relevant technology for the identification of wide variety of pathogen microorganisms (including *Candida* species) through their protein mass fingerprints [137-142]. VITEK® MS (bioMérieux) and MALDI Biotyper® (Bruker Daltonik GmbH) are MALDI-TOF MS instruments approved by the FDA for the diagnosis of IC (Figure 10) [50, 143]. These systems have several advantages. These take only 10-30 minutes to reach conclusive results from positive blood culture bottles, have high accuracy to diagnose IC, are associated with low reagent and labor costs, and allow microorganism identification at the species level [50, 137-138, 140, 144]. All these advantages have led to this proteomic technology replacing conventional methods of species-specific identification in most clinical mycology laboratories worldwide. However, these accurate, rapid and cost-effective platforms have also some

disadvantages. These mainly include their dependence on positive blood cultures (see above), spectral database limitations, and high instrument expense [35].

8.3. Immunomic methods

Different immunomics-based strategies, such as serological proteome analysis (SERPA), recombinant cDNA expression libraries (SEREX), and antigenic protein microarray assays, among others, have been developed to search for large panels of potential biomarkers for IC diagnosis and evaluate their clinical utility [97, 145-153]. SERPA (which combines high-resolution two-dimensional gel electrophoresis with Western blotting and mass spectrometry [154-156]) has allowed the identification and characterization of many antibody biomarker candidates for IC diagnosis, several of which have been validated in appropriate prototype immunoassays and have led to encouraging results [93, 98, 146, 157-159]. However, all these assays remain investigational and have not yet been implemented in clinical mycology laboratory.

9. Outlook

Over the past decades, a wide variety of rapid, easy and cost-effective methods for the detection and identification of *Candida* species in patients at risk for IC have emerged with the unique challenging purpose of minimizing the clinical impact and economic burden of this life-threatening and costly fungal infection. Despite all these enormous research and development efforts, none of these methods has shown to be perfect to diagnose IC nor have they proven to reduce the elevated mortality rates, length of hospital stay and healthcare costs attributable to IC. However, significant advances have been made in improvement of IC detection. More clinical

studies are needed for each assay in larger patient cohorts and many centers to determine its diagnostic performance in cases of deep-seated candidiasis (particularly in the absence of candidemia), assess its cost-effectiveness and associated management strategies in low and high-prevalence settings, compare its diagnostic accuracy with other assays, and evaluate the diagnostic utility of its combined use with other assays or clinical prediction algorithms, to name but a few. Future studies should also be aimed at searching for novel and better strategies that allow an early and accurate diagnosis of IC.

Disclosure of potential conflicts of interest

There are no conflicts of interest to disclosure. The funders had no role in study design, data collection, decision to publish, or preparation of the manuscript.

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Figure legends

Figure 1. Different cell morphologies of *Candida* species in infected tissues from IC patients.

Depending on *Candida* species, distinct morphological forms (yeasts, pseudohyphae and/or hyphae) can be found in clinical lesions from IC patients. All of them are pathogenic and can promote different stages of infectious process. *Asterisk* indicates that *C. krusei* (currently known as *P. kudriavzevii*) is now considered a member of the genus *Pichia* rather than the genus *Candida* [18]. CHROMagar™ Candida is a differential, chromogenic solid medium used for presumptive identification of clinically relevant *Candida* spp. from positive (blood) culture bottles. *Dagger* denotes that *C. auris* isolates show white to cream colored smooth colonies on CHROMagar™ Candida medium supplemented with Pal's agar, and can be discriminated from isolates identified as *Candida haemulonii* by other conventional method (Vitek® 2 YST ID card) [59]. See text for further information. *CFU*, colony-forming unit.

Figure 2. Overview of the current clinical setting for human IC.

IC remains a major infectious cause of morbidity and mortality in critically ill and severely immunocompromised patients, and further accounts for substantial healthcare costs. Data on its clinical impact and economic burden are from [1, 4, 8, 16-17, 34, 48, 161-163]. See text for further details. *BSI*, bloodstream infections; *HAI*, healthcare-associated infections.

Figure 3. Currently available methods for IC diagnosis.

Diverse methods have been developed and evaluated for IC diagnosis. These encompass the gold standards, nonculture laboratory tests based on measurement of pathogen and host-derived biomarkers, clinical prediction tools, and leading-edge technologies. See text for further information. *PCR*, polymerase chain reaction; *CAGTA*, *Candida albicans* germ tube antibody;

PCT, procalcitonin; *SAA*, serum amyloid A; *IL17*, interleukin 17; *PNA-FISH*, peptide nucleic acid-fluorescent in situ hybridization; *MALDI-TOF*, matrix-assisted laser desorption-ionization time-of-flight; *MS*, mass spectrometry.

Figure 4. Main advantages and disadvantages of the current gold standard methods for IC diagnosis.

The current gold standards to diagnose IC include fungal culture and tissue histopathology from normally sterile sites. The main advantage of these direct detection techniques of invasive fungal infection is their high specificity, whereas their major disadvantages are their low sensitivity, slow turnaround times (*except for tissue histopathology) and invasive testing (*except for blood culture). See text for further details.

Figure 5. Main pathogen and host-derived biomarkers for IC diagnosis.

Shaded rectangle shows the main commercially available tests for IC diagnosis based on measurement of *Candida* or human biomarkers. *Asterisk* indicates FDA-cleared assay to diagnose IC (see Figure 10). *Line with arrowhead at both ends* depicts those commercial diagnostic tests used in combination in clinical practice. See text for further information. *rRNA*, ribosomal RNA; *ERG11*, cytochrome P450 lanosterol 14-α-demethylase gene; *CHS1*, chitin synthase gene; *Hsp90*, 90-kDa heat shock protein; *Eno1*, enolase; *Mp65*, 65-kDa mannoprotein; *Sap1/2*, secreted aspartyl proteinase 1/2; *Ag*, antigen; *Ab*, antibodies; *CAGTA*, *Candida albicans* germ tube antibody; *IFA*, immunofluorescence assay; *IgG*, immunoglobulin G; *PCT*, procalcitonin; *SAA*, serum amyloid A; *IL17*, interleukin 17; *IL23*, interleukin 23; *TGF-ß*, transforming growth factor ß.

Figure 6. *Candida* cell wall polysaccharides as diagnostic biomarkers for IC.

A. Schematic representation of the *C. albicans* cell envelope. This encompasses an outer cell wall and an inner plasma membrane. The cell wall is basically composed of polysaccharides (mannan, ß-D-glucan and chitin) and proteins. Mannan and (1,3)-ß-D-glucan are its two most abundant constituents and major polysaccharide biomarkers for IC diagnosis, which can be detected by several commercial assays [1, 4]. See text for details. **B.** ESCMID-proposed recommendations about the use of gold standards, mannan/anti-mannan assays and ß-D-glucan assay for the diagnosis of candidemia, chronic disseminated candidiasis and other forms of IC in adults [82]. The use of mannan/anti-mannan assays is recommended in the diagnosis of candidemia and chronic disseminated candidiasis but not for other forms of IC, while the use of ß-D-glucan assay is recommended in the diagnosis of all these conditions.

Figure 7. *Candida* D-arabinitol as a diagnostic biomarker for IC.

L-arabinitol and D-arabinitol are enantiomers (optical isomers) that are present in human body fluids (urine and serum). Several medically important *Candida* species (*with the exception of *C. glabrata* and *C. krusei/P. kudriavzevi*) produce D-arabinitol (a five-carbon sugar alcohol). D-arabinitol quantification assays for IC diagnosis have been associated with false-positive results. The possible sources for false positivity (confounding factors) can be corrected by calculating relative amounts of D-arabinitol and L-arabinitol as well as D-arabinitol and creatinine (an indicator of renal dysfunction). *Brackets* represent concentration. See text for further details.

Figure 8. Mannan antigen and anti-mannan antibody quantification assays for IC diagnosis.

The combined use of both commercially available assays provides better discrimination between IC and non-IC patients than the individual assays alone. See text for further information. *Ag*, antigen; *Ab*, antibody; *HRP*, horseradish peroxidase; *mAb*, monoclonal antibody; *EBCA-1*, antibody directed against *Candida* (1-5)-α-oligomannosides; *pAb*, polyclonal antibody; *ELISA*, enzyme-linked immunosorbent assay.

Figure 9. Early diagnostic algorithm to identify patients at high risk for IC who may benefit from empirical antifungal therapy.

This algorithm is based on the combination of clinical scores with IC biomarkers (in particular, (1,3)-ß-D-glucan evaluated alone or in combination with CAGTA or procalcitonin, among other IC biomarkers, in two consecutive samples) [113, 126-127]. Empirical antifungal therapy is administrated in the absence of positive cultures in high-risk patients who manifest clinical signs and symptoms of infection [8]. See text for further details.

Figure 10. Comparison of the FDA-approved methods for IC diagnosis.

At present there are several methods cleared by the FDA to assist in the diagnosis of IC. These include culture-independent (Fungitell® and T2Candida®) and blood culture-dependent (Yeast Traffic Light® PNA-FISH®, VITEK® MS and MALDI Biotyper®) assays. *Asterisk* indicates that one of the five *Candida* species that can be detected with T2Candida® or Yeast Traffic Light® PNA-FISH® assays, *i.e. C. krusei* (currently known as *P. kudriavzevii*), is now considered a member of the genus *Pichia* rather than the genus *Candida* [18]. See text for further information. *FDA*, Food and Drug Administration; *T2RM*, T2 magnetic resonance; *PNA-FISH*, peptide nucleic acid-fluorescent in situ hybridization; *MALDI-TOF*, matrix-assisted laser

desorption/ionization time-of-flight; *MS*, mass spectrometry; *CFU*, colony-forming unit; *ITS2*, internal transcribed spacer 2; *rRNA*, ribosomal RNA.