Prediction of the Clinical Outcome in Invasive Candidiasis Patients Based on Molecular Fingerprints of Five Anti-Candida Antibodies in Serum*

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Better prognostic predictors for invasive candidiasis (IC) are needed to tailor and individualize therapeutic decision-making and minimize its high morbidity and mortality. We investigated whether molecular profiling of IgG-antibody response to the whole soluble Candida proteome could reveal a prognostic signature that may serve to devise a clinical-outcome prediction model for IC and contribute to known IC prognostic factors. By serological proteome analysis and data-mining procedures, serum 31-IgG antibody-reactivity patterns were examined in 45 IC patients randomly split into training and test sets. Within the training cohort, unsupervised two-way hierarchical clustering and principal-component analyses segregated IC patients into two antibody-reactivity subgroups with distinct prog-  

oses that were unbiased by traditional IC prognostic factors and other patients-related variables. Supervised discriminant analysis with leave-one-out cross-validation identified a five-IgG antibody-reactivity signature as the most simplified and accurate IC clinical-outcome predictor, from which an IC prognosis score (ICPS) was derived. Its robustness was confirmed in the test set. Multivariate logistic-regression and receiver-operating-characteristic curve analyses demonstrated that the ICPS was able to accurately discriminate IC patients at high risk for death from those at low risk and outperformed conventional IC prognostic factors. Further validation of the five-IgG antibody-reactivity signature on a multiplexed immunoassay supported the serological proteome analysis results. The five IgG antibodies incorporated in the ICPS made biologic sense and were associated either with good-prog- 

nosis and protective patterns (those to Met6p, Hsp90p, and Pgk1p, putative Candida virulence factors and anti-apoptotic mediators) or with poor-prognosis and risk patterns (those to Ssb1p and Gap1p/Tdh3p, potential Candida proapoptotic mediators). We conclude that the ICPS, with additional refinement in future larger prospective cohorts, could be applicable to reliably predict patient clinical-outcome for individualized therapy of IC. Our data further provide insights into molecular mechanisms that may influence clinical outcome in IC and uncover potential targets for vaccine design and immunotherapy against IC. Molecular & Cellular Proteomics 10: 10.1074/mcp. M110.004010, 1–26, 2011.

Despite recent advances in antifungal therapy, invasive candidiasis (IC) remains a leading infectious cause of morbidity and mortality in cancer, postsurgical, and intensive care patients (1–3). Its significant impact on patient clinical outcome, as reflected in its increased attributable mortality (10%–49%), length of hospital stay (3–30 days per patient), and healthcare costs (US $ 6214–92,266 per episode), could however be ame- liorated if early and appropriate antifungal therapeutic strategies were administered (1, 4). This precondition highlights the need to search for prognostic features that may reliably predict the clinical outcome in IC patients at presentation to tailor and individualize therapeutic decision-making accordingly and, as a result, to minimize the burden of the invasive infections caused by Candida spp. (commonly Candida albicans (1)).

Several factors have classically been reported to adversely influence the clinical outcome of IC patients (3, 5–7). None-theless, the prognostic potential of some of these traditional factors for IC is controversial (8, 9) and overall these have a limited prognostic power. For this reason, alternative laboratory tests based on measurement of Candida β-arabinotol/creatinine ratio, Candida antigen titer, or anti-Candida anti-  

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A 5-IgG Antibody-Reactivity Signature for IC Prognosis

Body levels (10–15) have been developed to explore their prognostic usefulness in IC. However, none of them has yet been validated for routine clinical practice. Furthermore, these few biomarkers may lack sensitivity for individual prediction of clinical outcomes in the first stages of infection and/or are not yet sufficiently accurate to attain widespread clinical use. In the light of these limitations, and considering the heterogeneity and intricacy of the host responses and molecular mechanisms underlying IC pathogenesis, it is likely that optimally combined multiple biomarkers may cover a broader range of IC patients and pathogenicity-related issues and more reliably predict IC prognosis in an early stage.

Serological proteome analysis (SERPA) may be a promising tool in this context because this global profiling technique enables the simultaneous assessment of reactivities of antibodies to a large panel of immunogenic proteins (i.e., the immunome of a (micro)organism (16)) in one experimental approach (17–21). This strategy has widely been applied to antibody-reactivity profiling for diagnostic and therapeutic purposes in cancers, autoimmune disorders, allergies, and infectious diseases (including IC (13, 15, 22, 23)) (18, 24–30). Despite that attractive clinical value, little is known, however, about the potential of this immunoproteomic method to identify antibody-reactivity patterns or signatures (18, 31) that may have utility in predicting the prognosis of individual patients with these pathologies. These prognostic signatures might further offer insights into IC pathogenesis and uncover potential targets for molecular therapies against IC. This approach could also profit from bioinformatics to search for hidden trends within generated multidimensional data and derive useful new knowledge (models, algorithms or rules) (32, 33).

Here, we examined the reactivity profiles of serum antibodies to the whole soluble *Candida* immunome at an early stage of IC by using SERPA and data-mining procedures in order to determine whether these could be indicative of distinct clinical outcomes in IC patients at presentation. We investigated whether these patterns could further reveal a prognostic signature that may serve to create a robust and consistent molecular predictor of clinical outcome for IC applicable to clinical practice and contribute to the traditional prognostic factors for IC. We then developed a multiplexed immunoassay to simultaneously and rapidly measure this simplified molecular fingerprint in each serum specimen and evaluate whether this could be a useful method for individual prediction of clinical outcomes in IC. We also explored whether this prognostic signature could yield biologic insights into molecular mechanisms that confer protection against IC and provide potential molecular targets for the design of novel vaccine- and/or immunotherapy-based strategies to prevent and control IC.

**EXPERIMENTAL PROCEDURES**

**Study Population and Serum Specimens**

Serum specimens from 48 adult IC patients belonging to different risk groups were obtained on the day of culture sampling at the Salamanca Clinic Hospital (Spain), a 750-bed tertiary care university-affiliated hospital, between December 1997 and March 2003. All patients were enrolled according to protocols approved by the Ethics Committee of Clinical Research from Salamanca Clinic Hospital, after informed consent was obtained. Patients were defined as having IC if they had clinical signs of infection or sepsis, and the same *Candida* species in one or more blood cultures and/or in cultures from at least three noncontiguous sites with an inadequate response to broad-spectrum antibiotics. Blood was cultured by the Bactec 9000 method (Becton Dickinson, Baltimore, MD), and specimens from other anatomic sites were plated on Sabouraud glucose agar. Yeast species were identified with the germ-tube formation test and the API-20C AUX system (BioMérieux, France). Three patients who had received antifungal drug prophylaxis before diagnosing IC were excluded from the study to ensure (i) the proper identification of potential prognostic biomarkers as these indicate the likely course of the disease in an “untreated” patient once the disease status has been established (15, 34, 35), and (ii) no overestimation or underestimation of the accuracy of our prediction model in patients who were responsive or nonresponsive, respectively, to the administered antifungal treatment given correlation between anti-*Candida* antibody levels and antifungal treatment efficacy (22, 36). The 45 specimens used in the present report were taken from an earlier retrospective case-control study (13). The outcome of hospital stay, death (n = 12) or discharge (n = 33), was recorded for each patient within 2 months. Details of baseline characteristics of the patients recruited in this retrospective study were described previously (13) and are shown in Table I on the basis of their clinical outcome.

Collection, processing and storage of serum specimens were uniform to avoid any systematic bias, and performed according to standard procedures. Briefly, blood samples were obtained by venepuncture using BD Vacutainer safety-lok blood collection set (Becton Dickinson) and collected in 10-ml BD Vacutainer tubes without additives (Becton Dickinson). They were allowed to clot at room temperature for 30 min and centrifuged at 3500 rpm for 10 min at room temperature. The upper phase (serum) of each specimen was then transferred to four 1.5-ml tubes, which were labeled with a code (to protect patient confidentiality) and kept at −80 °C. Serum samples were submitted to the Department of Microbiology II (Complutense University of Madrid, Spain) in dry ice.

Upon arrival, one tube of each sample was thawed on ice and divided into smaller aliquots using tubes for one-time use. These were labeled with the same code and stored at −80 °C. Serum aliquots from IC patients were thawed on ice just before analyses and assessed in a blinded fashion within 2 months following sample collection for the SERPA assay or at the present for the multiplexed immunoassay. We followed the proposed guidelines for reporting studies on clinical proteomics (37) and diagnostic accuracy (38).

**SERPA Assay**

**Preparation of Whole Soluble *C. albicans* Protein Extracts**—Whole soluble protein extracts of a clinical *C. albicans* isolate (strain SC3514) were used as an antigen source and prepared as reported (39, 40). Briefly, yeast cells were grown in YPD medium (1% Difco yeast extract, 2% peptone, and 2% glucose) at 30 °C up to an *A*$_{600}$ nm of 1.0, and washed with water. Cells were resuspended in cold lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 5 μg/ml each of leupeptin, pepstatin, and antipain (Sigma)) and lysed mechanically with an equal volume of glass beads in a fast-prep cell breaker (Q-Biogene, Carlsbad, CA). The clarified supernatant was stored at −80 °C. Protein concentration was measured with the Bradford assay (Bio-Rad, Hercules, CA).

**Two-Dimensional Polyacrylamide Gel Electrophoresis (2-DE)**—Whole soluble *C. albicans* protein extracts were separated by 2-DE as

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# Base-line characteristics of the study population in the training, test and entire-data sets according to the clinical outcome of IC patients within two months after presentation

| Characteristics                                      | Number (%) of IC patients |          |          |          |
|------------------------------------------------------|---------------------------|----------|----------|----------|
|                                                      | Good prognosis (n = 16)    | Poor prognosis (n = 6) | Good prognosis (n = 17) | Poor prognosis (n = 6) |
|                                                      | Training set (n = 22)      | Test (validation) set (n = 23) | Entire-data set (n = 45) |

## Demographic factors
- **Sex**
  - Male: 7 (43.8), 5 (83.3), 12 (70.6), 4 (66.7), 19 (57.6), 9 (75.0)
  - Female: 9 (56.3), 1 (16.7), 5 (29.4), 2 (33.3), 14 (42.4), 3 (25.0)

## Age (years)
- ≤49 years: 3 (18.8), 0 (0.0), 5 (29.4), 3 (50.0), 8 (24.2), 3 (25.0)
- 50-69 years: 6 (37.5), 4 (66.7), 10 (58.8), 2 (33.3), 16 (48.5), 6 (50.0)
- ≥70 years: 7 (43.8), 2 (33.3), 2 (11.8), 1 (16.7), 9 (27.3), 3 (25.0)

## Comorbidities
- **Malignancies**
  - Hematological malignancy: 6 (37.5), 1 (16.7), 7 (41.2), 2 (33.3), 13 (39.4), 3 (25.0)
  - Solid tumor: 5 (31.3), 3 (50.0), 2 (11.8), 1 (16.7), 7 (21.2), 4 (33.3)

## Nonmalignant diseases
- **Respiratory dysfunction**
  - 2 (12.5), 1 (16.7), 3 (17.6), 2 (33.3), 5 (15.2), 3 (25.0)

## Gastrointestinal pathology
- 1 (6.3), 1 (16.7), 4 (23.5), 0 (0.0), 5 (15.2), 1 (8.3)

## Others
- 2 (12.5), 0 (0.0), 1 (5.9), 1 (16.7), 3 (9.1), 1 (8.3)

## Risk factors for mortality in IC
- **Older age (>65 years)**: 9 (56.3), 3 (50.0), 7 (41.2), 2 (33.3), 16 (48.5), 5 (41.7)
- **Leukocytosis**: 4 (25.0), 3 (50.0), 6 (35.3), 3 (50.0), 10 (30.3), 6 (50.0)

## Presence or persistence of neutropenia
- 4 (25.0), 2 (33.3), 3 (17.6), 11 (64.7), 7 (21.2), 3 (25.0)

## Underlying malignant condition
- 11 (68.8), 4 (66.7), 9 (52.9), 3 (50.0), 20 (60.6), 7 (58.3)

## Immunosuppressive therapy
- 5 (31.3), 4 (66.7), 3 (17.6), 4 (66.7), 8 (24.2), 8 (66.7)

## Recent major surgery
- 3 (18.8), 3 (50.0), 5 (29.4), 2 (33.3), 8 (24.2), 5 (41.7)

## Hematopoietic transplantation
- 2 (12.5), 1 (16.7), 1 (5.9), 1 (16.7), 3 (9.1), 2 (16.7)

## Central venous catheters
- 6 (37.5), 2 (33.3), 5 (29.4), 2 (33.3), 11 (33.3), 4 (33.3)

## Intensive care unit stay
- 2 (12.5), 3 (50.0), 4 (23.5), 4 (66.7), 6 (18.2), 7 (58.3)

## Inappropriate initial antifungal treatment
- 2 (12.5), 0 (0.0), 1 (5.9), 1 (16.7), 3 (9.1), 1 (8.3)

## Diabetes mellitus
- 2 (12.5), 1 (16.7), 1 (5.9), 1 (16.7), 3 (9.1), 2 (16.7)

## Adult respiratory distress syndrome (ARDS)
- 2 (12.5), 2 (33.3), 2 (11.8), 2 (33.3), 4 (12.1), 4 (33.3)

## Acute renal failure
- 1 (6.3), 1 (16.7), 1 (5.9), 1 (16.7), 2 (6.1), 2 (16.7)

## Sepsis or septic shock at presentation
- 1 (6.3), 2 (33.3), 3 (17.6), 1 (16.7), 4 (12.1), 3 (25.0)

## Recent use of broad-spectrum antibiotics
- 8 (50.0), 4 (66.7), 11 (64.7), 6 (100.0), 19 (57.6), 10 (83.3)

## Pneumonia or other concomitant bacterial infection
- 4 (25.0), 2 (33.3), 5 (29.4), 3 (50.0), 9 (27.3), 5 (41.7)

## Causative Candida species
- C. albicans: 14 (87.5), 4 (66.7), 9 (52.9), 4 (66.7), 23 (69.7), 8 (66.7)
- Others: 2 (12.5), 2 (33.3), 8 (47.1), 2 (33.3), 10 (30.3), 4 (33.3)

## Inappropriate initial antifungal treatment
- 2 (12.5), 1 (16.7), 1 (5.9), 1 (16.7), 3 (9.1), 2 (16.7)

## Hospital wards
- Intensive care unit: 2 (12.5), 3 (50.0), 4 (23.5), 4 (66.7), 6 (18.2), 7 (58.3)
- Hematology-oncology unit: 5 (31.3), 1 (16.7), 7 (41.2), 1 (16.7), 12 (36.4), 2 (16.7)
- General medicine wards: 9 (56.3), 2 (33.3), 6 (35.3), 1 (16.7), 15 (45.5), 3 (25.0)

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*a* Training and test sets were balanced for mortality rate (see Experimental Procedures for further details).

*b* IC patients who died within two months of Candida isolation.

*c* Only the primary condition is shown.

*d* Includes the following diseases: leukemia, lymphoma, myelodyplasia, and multiple myeloma.

*e* Includes the following diseases: bronchopulmonary neoplasm, pancreas/colon adenocarcinomas, and bladder neoplasm.

*f* Includes the following diseases: pneumonia, chronic obstructive pulmonary disease, and adult respiratory distress syndrome.

*g* Includes the following diseases: cholecystitis, angiocholitis, pancreatitis, peritonitis, and hepatitis.

*h* Include the following diseases: multiple trauma, acute renal insufficiency, and diabetes mellitus.

*i* Main risk factors for hospital mortality in IC patients are shown (3, 5).

*j* Leukocytosis was defined as a white blood cell count above 11,000 cells/mm³.

*k* Neutropenia was defined as an absolute neutrophil count below 500 cells/mm³.

*l* Includes systemic corticosteroids (≥7.5 mg prednisone per day or equivalent), immunosuppressive or cytotoxic drugs and/or total-body irradiation. All patients with systemic corticosteroid therapy received 1–3 mg prednisone per kilogram per day for >15 days.

*m* p < 0.05 for the comparison with the good-prognosis test group.

*n* p < 0.05 for the comparison with the good-prognosis entire-data group.

*o* Includes colectomy, duodenotomy, cholecystectomy, pancreatectomy, pulmonary lobectomy, and thoracotomy.

*p* Diabetes mellitus and ARDS were not the primary condition in most IC patients.

*q* A slight, but not statistically significant, difference (p = 0.06) was observed for the comparison with the good-prognosis training group.

# Table I

| Characteristics                                      | Number (%) of IC patients |          |          |          |
|------------------------------------------------------|---------------------------|----------|----------|----------|
|                                                      | Good prognosis (n = 16)    | Poor prognosis (n = 6) | Good prognosis (n = 17) | Poor prognosis (n = 6) |
|                                                      | Training set (n = 22)      | Test (validation) set (n = 23) | Entire-data set (n = 45) |

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described elsewhere (22, 41), using immobilized, nonlinear pH 3–10 gradient strips (18 cm; GE Healthcare, Buckinghamshire, UK) for isoelectric focusing, and 10% SDS-polyacrylamide gels (10% T; 1.6% C) for the second-dimension separation. The 2-DE-separated proteins were then either visualized with silver nitrate (42, 43) or colloidal Coomassie Brilliant Blue (40, 44) or electrotransferred to nitrocellulose membranes (40).

Two-Dimensional Western Blot Analysis—Two-dimensional Western blot assay was carried out essentially as reported (13, 22, 40). Serum anti-Candida IgG antibody levels were indirectly measured in each screened sample by densitometric analysis of the corresponding Western-blotting results using the ImageMaster 2D Platinum software (GE Healthcare) and expressed as arbitrary units relative to the integrated optical density of their related spot area following background subtraction and normalization analyses as described previously (13, 40).

Mass Spectrometry (MS) Analysis—Protein spots of interest that were not identified using our reference two-dimensional map of C. albicans immunogenic proteins (22) (also available on our COMPLU-YEAST-2DPAGE database at http://www.expasy.ch/ch2d/2d-index.html (45, 46)) were manually excised from silver or colloidal Coomassie-stained preparative 2-DE gels, and in-gel destained, reduced, alkylated, and digested with trypsin (42, 47). The resulting tryptic digests were analyzed on a matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometer (Voyager-DE STR, PerSeptive Biosystems, Framingham, MA). All MS spectra were calibrated externally with the Sequazyme peptide mass standard kit (PerSeptive Biosystems, Framingham, MA) and internally using trypsin auto-digestion products.

Raw data were processed using the Data Explorer software v.4.5 (Applied Biosystems, Framingham, MA) prior to searching with the Mascot search engine. The parameters used to analyze the data were: signal-to-noise threshold of 20, a minimum area of 100, a resolution of over 10,000, and exclusion of all known contaminant ions (including keratins). Protein identification from processed spectrum data was performed on a local Mascot server v.1.9 using CandidaDB (2003.05.22; 6165 sequences; 2952183 residues) and Candida Genome Database (CGD) (2007.01.31; 6107 sequences; 2981547 residues) under the following search parameters: enzyme, trypsin; fixed modifications, S-carbamidomethylation of Cys; variable modifications, oxidation of Met; mass values, monoisotopic; peptide tolerance, 50 ppm; and number of missed cleavage sites, up to 1. No relative molecular mass or isoelectric point constrictions were used. Identifications were accepted as positive when there were at least five matching peptide masses with 25–50 ppm mass accuracy, at least 20% sequence coverage, and a significant gap between the first and the next best (false-positive) database hits. Protein scores greater than 50 were significant (p < 0.05).

Production of Recombinant C. albicans Proteins

Cloning—The MET6, SSB1, GAP1/TDH3, HSP90 and PKG1 coding sequences flanked with Sall and Nol sites were PCR-amplified from C. albicans SC5314 genomic DNA using the primers reported in Supplemental Table S1. The PCR products were cloned into the pGEM-T vector (Promega, Madison, WI), and the recombinant plasmids were transformed into Escherichia coli DH5α. The inserts were subcloned into the Sall and Nol sites of the E. coli pGEX-6P-3 vector (GE Healthcare) in-frame with the glutathione-S-transferase (GST) coding sequence. The plasmids were then transformed into E. coli BL21 for expression of fusion proteins.

Protein Expression—Single colonies of selected recombinants were grown in LB medium (0.5% Difco yeast extract, 1% tryptone, and 1% NaCl) at 37 °C up to an A600 nm of 0.6–0.7. Protein expression was then induced by adding isopropyl-β-D-thiogalactoside to a final concentration of 1 mM. After growing for another 12 h at 24 °C (for Met6p, Ssb1p and Hsp90p) or 3 h at 37 °C (for Gap1p/Tdh3p and Pgp1p), cells were resuspended in precooled lysis buffer (PBS containing 0.1% lysozyme, 1% Triton X-100 and 1 mM phenylmethylsulfonyl fluoride) and sonicated on ice for 15–60 s. Expression of GST fusion proteins was monitored before and after induction using SDS-PAGE followed by Coomassie blue staining (44) and Western blotting using anti-GST antibodies (1:5000 dilution, Santa Cruz Biotechnology, Santa Cruz, CA).

Protein Purification—The GST-tagged recombinant proteins were purified using glutathione Sepharose beads (4B, GE Healthcare) and treated with the Pre-Scission protease (GE Healthcare) to remove the GST moiety according to the manufacturer’s instructions.

Protein Characterization—The identity of the purified proteins was confirmed by MS analysis as aforementioned, with slight modifications. Briefly, following trypsin digestion, the resulting peptides were analyzed using the 4800 Plus MALDI-TOF/TOF Analyzer (Applied Biosystems) and the 4000 Series Explorer software v.3.5.3 (Applied Biosystems). MALDI-TOF spectra were internally calibrated with trypsin auto-digestion products. MS peak filtering was performed through the Global Protein Server Explorer software v.3.6 (Applied Biosystems) using the same parameters and thresholds as cited above. Database searches of processed MS data for protein identification were carried out through the global protein server Explorer software on a local Mascot server v.2.1 using CandidaDB and CGD (2003.05.22 and 2007.01.31; 6165 and 6107 sequences; 2981547 and 2925183 residues, respectively) and the same search parameters and acceptance criteria as described above. Preservation of antigenic properties of the recombinant proteins was verified by SDS-PAGE Western-blotting using three IC specimens with different serum levels of IgG antibodies to them as reported previously (23).

Multiplexed Immunoassay for the Best Predictor Variables

Multiplexed Dot-Blot Assay—For each immunoarray, 0.5 μg of each purified recombinant protein, bovine serum albumin (negative control) (Sigma), and C. albicans protein extract (positive control) were spotted on a nitrocellulose membrane (GE Healthcare). Blots were first visualized by transient Ponceau S staining (Serva, Heidelberg, Germany), scanned, and rinsed with TBS. They were then blocked with 5% nonfat dry milk in TBS for 2 h, washed with TBS containing 0.01% Tween-20 (TTBS), and incubated with serum samples at a 1:500 dilution (or at a 1:250–1:1000 dilution whether the initial relative dot volume value was not within the log-linear interval of the calibration curve) in assay buffer (1% nonfat dry milk in TTBS) for 2 h. Following rinsing, immunoblots were incubated with horseradish peroxidase-labeled anti-human IgG antibodies (Abbott Healthcare) at a 1:3000 dilution in assay buffer for 1 h, and washed again. Immuno-blots were revealed using the enhanced chemiluminescence detection system (GE Healthcare). Thereafter, densitometric analyses were performed on them using the Quantity-One software (Bio-Rad). The concentration of each signature IgG antibody in each serum was calculated as the relative volume of its corresponding immunoreactive protein dot following background subtraction and normalization to the loading control (Ponceau-stained blots), and expressed in reference units (RU)/ml relative to threefold serial dilutions of a serum pool from one IC survivor and one IC nonsurvivor (calibrator) with 6000 RU/ml. Samples were tested on three independent experiments except where noted otherwise.

Analytical Validation—The limit of detection was calculated as the signature IgG antibody concentration corresponding to a signal 2.5 S.D. above the mean of 24 replicates of the zero calibrator (assay...
buffer). Imprecision was estimated for three serum samples (with different signature antibody concentrations within the assay range) tested in four replicates per run per day for six nonconsecutive days. Imprecision profiles were determined at different calibrator concentrations assayed on six nonconsecutive days. The limit of quantification was defined as the minimum signature IgG antibody concentration with an interassay imprecision ≤20%. The assay linearity was established in six serum specimens serially diluted from 1:250 to 1:1000 in the assay buffer. Analytical recovery was examined in six serum samples assayed before and after addition of 0.5, 1.5, and 2.5 RU/ml signature IgG antibodies. Recovery was calculated as the ratio of recovered to expected concentrations, and expressed as a percentage.

Gene Ontology (GO) Classification

The GeneCoDis web-based tool v.2 (http://genecondis.dacya.ucm.es) (48) was used to assess whether certain GO categories and their combinations were over-represented among the genes encoding C. albicans proteins recognized by IgG-antibody signatures. GO analyses were performed by comparing the number of genes in a defined input list (related to IgG-antibody signatures) that fall into a GO to the number of genes in the reference list (the entire C. albicans genome at the CGD database) that fall into the same ontology. Over-representation of selected GO categories (biological processes and molecular functions) and their combinations in the input gene lists was determined using the hypergeometric test with the stimulation-based correction.

Statistical Analysis

Training and test sets were randomly generated using the Bernoulli distribution with the specified probability parameter 0.5 following sample prestratification to ensure a similar number of patients with good and poor prognoses in each group. The Kolmogorov-Smirnov test and Shapiro-Wilk W-test were applied to determine whether data conformed to a normal distribution. Categorical variables were evaluated using the Yates’ corrected $\chi^2$ test or Fisher’s test, as appropriate. The pairwise group and multiple comparisons of continuous data were examined using the Mann-Whitney test and the Kruskal-Wallis test, respectively. Paired observations were compared with the Wilcoxon signed-rank test. Correlations were estimated by the Spearman’s rank-correlation coefficient. In the multiplexed immunoassay, nonlinear-regression analyses using a four-parameter logistic function were applied for calibration curves. Imprecision profiles were established using quadratic-regression models. Linear-regression analyses were performed on dilution linearity and analytical recovery assays.

Two-way hierarchical clustering analysis (HCA) and principal-component analysis (PCA) were used to group the serum samples according to the similarity of their IgG antibody-reactivity patterns (49, 50). IgG antibody levels were normalized by median-centering antibodies for each sample and then by median-centering of each antibody across all samples. The Mann-Whitney test and analysis of variance were applied on PCA data to assess the homology and homogeneity, respectively, of the antibody-reactivity profiles among groups. Distances among clinical and molecular prognostic indicators for IC were examined by one-way HCA and PCA.

Discriminant analysis was applied to model the relationship between the clinical outcome of IC patients and their anti-Candida IgG antibody patterns as well as between patient outcomes and traditional IC prognostic factors, and to find a linear combination of the minimum number of predictor variables that best separated the compared groups (classification algorithm or discriminant function). The colinearity degree among the predictor variables was estimated with the tolerance level. Correlations between predictor variables were visualized with the within-groups correlation matrix. The Box’s M test was used to determine whether covariances across groups were equal. The contribution of each predictor variable to the discriminant model was assessed by canonical correlation analysis using the equality tests of group means, the discriminant function coefficients, and the combined within-groups correlations between discriminant variables and functions. The best predictor variables to incorporate in the discriminant model were selected with the stepwise method based on the $F$ statistic. Correlation between the discriminant scores and the groups was estimated by the canonical correlation coefficient. The proportion of the total variance in the discriminant scores not explained by differences among groups was calculated with the Wilks’ $\lambda$ statistic. The class prediction accuracy and error of the discriminant model were calculated by means of the $U$-method or leave-one-out cross-validation (LOOCV) within the training set, followed by further validation within the test set.

Receiver-operating-characteristic (ROC) curves were used to examine the discriminative ability of the prediction models. ROC areas were compared with a nonparametric asymptotic method (51). Model cutoff thresholds were defined on the basis of ROC curve analysis on training set data. These discrimination threshold values correspond to the highest combined sensitivity and specificity (i.e. the minimum sum of false-negative plus false-positive test results) for poor or good clinical outcomes when mortality or survival predictors, respectively, for IC were determined. The exact 95% confidence intervals (CIs) for the test operating characteristics of the prognostic predictors were calculated on the basis of a binomial approximation to the normal distribution. The degree of agreement between prediction models was established using the Cohen’s kappa statistic. Odds ratios were estimated to determine the association between IgG antibody patterns and clinical outcomes in IC. Values below the 25th percentile of the level distribution of IgG antibodies to Met6p, Hsp90p and Pkg1p in IC survivors or to Ssb1p and Gap1p/Tdh3p in IC nonsurvivors were considered low levels. Multivariate logistic-regression models were performed to control potential confounding factors and to identify independent prognostic predictors for IC. Model goodness-of-fit was assessed using the Hosmer-Lemeshow test. Statistical significance was set at $p < 0.05$ (two-sided).

RESULTS

Anti-Candida IgG Antibody-Reactivity Profiles of IC—To devise an antibody reactivity-based prognostic predictor for IC, serum samples from 45 IC patients with favorable ($n = 33$) and fatal ($n = 12$) clinical outcomes within 2 months following presentation were individually screened by SERPA for IgG antibodies to the whole soluble C. albicans immunome, with the patients randomly, but equally, split into a training set for prediction-model development and into a test set for model validation (Fig. 1). Both sets, balanced for mortality, did not significantly differ in their baseline characteristics (Table I). Protein spots detected in at least three training IC patients were identified by MS analysis or using our reference two-dimensional map (22). In all, 31 C. albicans proteins, 21 of which showed several protein species on the two-dimensional recognition profiles (Fig. 2), from six functional groups were characterized as elicitors of early antibody responses in IC patients (Table II and Supplemental Fig. S1). Similar two-dimensional antibody-reactivity patterns and consistent recognition intensities for this antigen panel (coefficient of variation, 3.8%–13.6%) were obtained with the 45 serum
specimens assessed on two independent experiments, indicating good reliability of the SERPA assay.

Within the training set, the overall reactivity profiles of IgG antibodies to these 31 Candida proteins were examined by unsupervised clustering analyses to determine whether there was an evident relationship among samples and underlying antibody-reactivity patterns (Fig. 3). Intriguingly, unsupervised two-way HCA revealed separate highly correlated antibody-reactivity profiles (antibody-reactivity signatures) that, without any prior statistical test to select prognosis-associated antibodies, segregated the 22 training specimens into two major clusters corresponding to patients with distinct clinical outcomes \( (p < 0.001; \text{Fig. 3A}) \). To elucidate the potential effects of traditional IC prognostic factors and other baseline characteristics on these antibody-reactivity subgroups, their distribution was next explored among samples. No significant associations were found between these variables and antibody-reactivity patterns (Fig. 3B), suggesting that these subgroups basically reflect differences in patient outcomes not outlined by these variables.

Unsupervised PCA was then performed on these antibody-reactivity profiles in order to confirm HCA results and support the robustness of these two classes. PCA demonstrated that these antibody-reactivity patterns were discrete and heterogeneous \( (p < 0.001) \) between the clinical-outcome subgroups but similar and homogeneous \( (p > 0.05) \) between the different subsets defined by classical IC prognostic factors (Fig. 3C).
Creation of an IgG Antibody Reactivity-Based Prognostic Predictor for IC—Having shown the presence of anti-Candida IgG antibody-reactivity signatures in serum at an early stage of IC that significantly correlated with the prognosis of IC patients by unsupervised methods, we next sought to develop a molecular predictor of clinical outcome for IC based on these antibody-reactivity profiles. To select the most relevant and critical panel of IgG antibodies for the prognostic signature and create a practical scoring system, the reactivity levels of the 31 IgG antibodies (predictor variables) in the training set were modeled as categorical variables (dichotomized as detectable versus undetectable, given good correlation between their seroprevalence and reactivity signatures; Table II) by supervised discriminant analyses (Fig. 1). A step-wise multivariate discriminant model containing five IgG antibodies (those to Met6p, Ssb1p, Gap1p/Tdh3p, Hsp90p, and Pgk1p; Fig. 4A) was identified, after ruling colinearity out among them, as the best prognostic predictor for IC (canonical correlation, 0.95; p < 0.001). This serum five-IgG antibody signature accounted for 91% of total variance in the reactivity profiles between survivors and nonsurvivors (Wilks’ λ, 0.09; χ², 41.63; p < 0.001).

Among the predictor variables included in the model, IgG antibodies to Met6p showed the highest discriminating ability, followed in order by those to Ssb1p, Gap1p/Tdh3p, Hsp90p, and Pgk1p (Figs. 4B and 4C). Combined within-group correlations of predictor variables with the standardized canonical discriminant functions highlighted that elevated seropreva-
TABLE II
Serum anti-Candida IgG antibody signature associated with IC and seroprevalence of these antibodies according to the clinical outcome in IC patients within the training set

| Main Candida immunogenic proteins (Candida immunome) | Serum IgG antibodies to the specified Candida immunogenic protein | % Difference (95% CI) in seroprevalence (good versus poor prognosis) | Predictive strength | Prognosis signature |
|-----------------------------------------------------|---------------------------------------------------------------|------------------------------------------------------------------|--------------------|---------------------|
| **I. Cell rescue, defense and virulence**           |                                                               |                                                                   |                    |                     |
| a. Stress response                                  |                                                               |                                                                   |                    |                     |
| Hsp90p                                              | 90kDa-Heat shock protein                                      | CA4959 82 4.48                                                    | 38 (11 to 64)      | 2.05 Good           |
| Ssa4p/Hsp70p                                        | Heat shock protein of the Hsp70p family                       | CA1230 70 4.71                                                    | −50 (−77 to −23)   | 2.96 Poor           |
| Ssb1p                                               | Heat shock protein of the Hsp70p family                       | CA3534 66 4.86−5.03                                              | −58 (−102 to −15)  | 1.94 Poor           |
| Ssc1p                                               | Heat shock protein of the Hsp70p family                       | CA4474 68 4.81−4.96                                              | −25 (−72 to 22)    | 0.55 Poor           |
| Sse1p/Msi3p                                         | Heat shock protein of the Hsp70p family and Hsp90p co-chaperone | CA1911 80 5.13−5.18                                             | 38 (11 to 64)      | 2.05 Good           |
| Ipf17186p/Hsp31p                                     | Unknown function; ortholog Saccharomyces cerevisiae 31-kDa heat shock protein | CA0828 26 4.32−4.40                                             | 10 (−41 to 62)     | 0.17 Good           |
| b. Detoxification and stress response               |                                                               |                                                                   |                    |                     |
| Grp2p                                               | Reductase; similar to plant dihydroflavonol-4-reductases      | CA2644 37 5.95−6.31                                              | 27 (−19 to 73)     | 0.65 Good           |
| Ipf2431p/Tsa1p                                      | Similar to S. cerevisiae thiol-specific antioxidant protein   | CA5714 20 4.72                                                   | 2 (−38 to 42)      | 0.04 Good           |
| **II. Carbon compound and carbohydrate metabolism** |                                                               |                                                                   |                    |                     |
| a. Glycolysis/gluconeogenesis pathway               |                                                               |                                                                   |                    |                     |
| Pgi1p                                               | Glucose-6-phosphate isomerase                                 | CA3559 58 6.33                                                   | −19 (−68 to 31)    | 0.36 Poor           |
| Fba1p                                               | Fructose biphosphate aldolase                                 | CA5180 39−40 5.85−6.00                                           | 27 (−19 to 73)     | 0.65 Good           |
| Tpi1p                                               | Triose phosphate isomerase                                    | CA5950 26−27 5.59−5.85                                           | −8 (−55 to 38)     | 0.15 Poor           |
| Gap1p/Tdh3p                                         | Glyceraldehyde-3-phosphate dehydrogenase                      | CA5892 35 6.67−7.38                                              | −48 (−91 to −5)    | 1.49 Poor           |
| Pgk1p                                               | Phosphoglycerate kinase                                       | CA1691 44−46 5.40−6.12                                           | 17 (−26 to 60)     | 0.44 Good           |
| Gpm1p                                               | Phosphoglycerate mutase                                       | CA4671 27−28 5.73−5.86                                           | 31 (6 to 57)       | 1.71 Good           |
| Eno1p                                               | Enolase                                                       | CA3874 48−49 5.25−5.65                                           | 0                   | n.a. / Good         |
| Cdc19p                                              | Pyruvate kinase                                               | CA3483 62 6.64−6.89                                              | −40 (−86 to −7)    | 1.07 Poor           |
| b. Fermentation pathway                            |                                                               |                                                                   |                    |                     |
| Pdc11p                                              | Pyruvate decarboxylase                                        | CA2474 60−63 5.00−5.22                                           | 38 (−3 to 78)      | 1.18 Good           |
| Adh1p                                               | Alcohol dehydrogenase                                         | CA4765 44−45 5.56−5.83                                           | −35 (−84 to 13)    | 0.84 Poor           |
| c. Glyoxylate and tricarboxylic acid (TCA) cycles   |                                                               |                                                                   |                    |                     |
| Aco1p                                               | Aconitase hidratase                                           | CA3546 84−85 5.82−5.90                                           | 25 (1 to 49)       | 1.39 Good           |
| Mdh1p                                               | Mitochondrial malate dehydrogenase                           | CA5164 35 5.56                                                   | 21 (−25 to 67)     | 0.47 Good           |
| d. Pentose phosphate pathway                        |                                                               |                                                                   |                    |                     |
| Tk1p                                                | Transketolase                                                | CA3924 73 5.54−5.59                                              | −15 (−58 to 29)    | 0.31 Poor           |
| **III. Amino acid metabolism**                      |                                                               |                                                                   |                    |                     |
| Met6p                                               | Methionine synthase                                           | CA0653 84−85 5.27−5.55                                           | 77 (47 to 107)     | 4.57 Good           |
| Shm2p                                               | Serine hydroxymethyl-transferase                             | CA0895 53 7.29                                                   | 2 (−42 to 46)      | 0.04 Poor           |
| Leu1p                                               | 3-Isovalerylmalate dehydrogenase                             | CA5942 91 5.63                                                   | 19 (−3 to 40)      | 1.08 Good           |
| **IV. Nucleotide, nucleoside and nucleobase metabolism** |                                                               |                                                                   |                    |                     |
| Ade17p                                              | 5-Aminomimidazole-4-carboxamide ribotransformylase            | CA4513 67 6.28−6.70                                              | −31 (−76 to 13)    | 0.80 Poor           |
| Imh3p                                               | Inosine-5′-monophosphate dehydrogenase                       | CA1246 57 6.67                                                   | −38 (−95 to 20)    | 0.78 Poor           |
| **V. Cofactor, prosthetic group and vitamin metabolism** |                                                               |                                                                   |                    |                     |
| Hem13p                                              | Coproporphyrinogen III oxidase                               | CA0517 36 5.83                                                  | −27 (−81 to 27)    | 0.58 Poor           |
| Ach1p                                               | Acetyl-coenzyme-A hydrolase                                  | CA0345 66 7.00                                                   | 8 (−35 to 52)      | 0.16 Good           |
TABLE II—continued

| Name | Function or homology | Candida DB accession number | Mr | pI | % Difference (95% CI) in seroprevalence (good versus poor prognosis) | Predictive strength | Prognosis signature |
|------|----------------------|-----------------------------|----|----|----------------------|-------------------|-------------------|
| VI. Protein synthesis | | | | | | | |
| a. Elongation factors | | | | | | | |
| EF1αp/CEF3p | Translation elongation factor 3 | CA3081 | 117 | 5.39–5.45 | 25 (1 to 49) | 1.39 | Good |
| EF2p | Translation elongation factor 2 | CA2810 | 93 | 6.55–6.72 | 2 (–42 to 46) | 0.04 | Poor |
| b. Ribosomal enzymes | | | | | | | |
| Bel1p/Asc1p | 40S small subunit ribosomal protein | CA4588 | 31 | 6.07 | –4 (–40 to 32) | 0.09 | Poor |

Immunogenic proteins were identified either by using our published reference two-dimensional map of (MS-characterized) C. albicans proteins specifically recognized by serum IgG antibodies from IC patients (22) (also available on our electronic COMPLUYEAST-2DPAGE database (45, 46)) or by using MALDI-TOF MS analyses (see Ssa4p/Hsp70p, Grp2p, Lpf2431p/Tsa1p, and Supplemental Fig. S1).

Protein names and functions/homologies according to CandidaDB and CGD (Candida Genome Database) web resources. When protein names differ in both databases, the first and second names correspond to those from CandidaDB and CGD, respectively, except where noted otherwise (see Ipf17186p/Hsp31p). Proteins are clustered into six functional groups in keeping with the comprehensive yeast genome database (CYGD) at MIPS (http://mips.gsf.de/proj/yeast/CYGD/db/).

Experimental Mr and pI values (calculated by using the ImageMaster 2D Platinum software).

Represents the percentage difference in the seroprevalence of the specified IgG antibody in IC patients who had a good prognosis minus that in IC patients who had a poor prognosis. A positive value thus signifies that its seroprevalence is higher in IC patients who had a favorable clinical outcome than in those who had a fatal clinical outcome, and vice versa.

Corresponds to the statistical weight of the percentage difference in the seroprevalence of each specified IgG antibody in good- vs. poor-prognosis groups, and was computed by using the negative natural log of the p value.

Overall, a good correlation was found between the seroprevalence of each specified IgG antibody and its reactivity signature (as determined by unsupervised HCA). See Fig. 3A for further details.

Protein identified by using MALDI-TOF MS analysis. See Supplemental Fig. S1 for further information on Mascot score, matched/unmatched peptides and protein coverage, among others.

Protein name in parenthesis is according to SGD (S. cerevisiae Genome Database) web resource, and corresponds to the S. cerevisiae ortholog.

Gap1p/Tdh3p is also involved in pentose phosphate pathway.

All IC patients (with good and poor prognoses) were seropositive for anti-Eno1p IgG antibodies. n.a. denotes not applicable.
lence of IgG antibodies to Met6p, Hsp90p, and Pgk1p was associated with good prognosis whereas high frequency of those to Ssb1p and Gap1p/Tdh3p correlated with poor prognosis (Fig. 4C). Supervised univariate logistic-regression analyses demonstrated that these five IgG antibodies associated with good- or poor-prognosis signatures were also related to protective or risk patterns, respectively; i.e. those to Met6p, Pgk1p, and Hsp90p were protective antibodies whereas those to Ssb1p and Gap1p/Tdh3p were risk (nonprotective) antibodies (Table III).

On the basis of the relative contributions of each of the five IgG antibodies in the discriminant model, we built a classification algorithm (IC prognosis score or ICPS) that provided each IC patient the likelihood of having a fatal clinical outcome in the ensuing two-month period. The median ICPS was significantly higher among IC patients who had a benign clinical course than among those who had an unfavorable outcome (2.22 versus −5.60; p < 0.001; Fig. 4D). An ICPS ≤−1.67 (defined by ROC plots) among the training IC patients was strongly predictive of a poor prognosis within 2 months (p < 0.001). The prognostic accuracy of this five-IgG antibody signature for IC was excellent (c-statistic, 1.00). It correctly classified 100% (22/22) of the training patients according to their clinical outcomes using this ICPS cutoff.

Validation of the Prognostic Predictor for IC—In an attempt to minimize the possible overestimation of the prognostic value of this molecular predictor (because of overoptimistic trend of classifications based on the cases used to create the prediction model (52)), we next tested its ability to accurately predict the prognosis of an unknown sample (not involved in the selection of predictor variables) using LOOCV within the training cohort (Fig. 1). For this class prediction analysis, one of the 22 training specimens was removed and a new prognostic predictor for IC was constructed, as described above, on the 21 remaining samples and then used to predict the clinical outcome of the left-out sample. Once this process was cycled through all of these specimens one by one, we found that the molecular fingerprint of the five IgG antibodies used in the original model displayed the highest recurrence over left-out iterations. This serum signature was able to correctly predict the true clinical outcome of 21 (95%; 95% CI, 87%–100%) out of the 22 iteratively left-out samples (classification error rate, 5%; 95% CI, 3%–7%; Figs. 5A and 5B, left), with 83% sensitivity (1 out of 6 nonsurvivors was misclassified) and 100% specificity (all survivors were correctly classified) for early identifying a fatal clinical outcome in the training set using an ICPS threshold of −1.67 (Table IV). Strikingly, the ICPS provided good LOOCV discriminatory power in this patient cohort (ROC area, 0.97; 95% CI, 0.90–1.00; p = 0.001; Fig. 5C, left).

We then assessed the robustness and consistency of this clinical-outcome prediction model for IC in the test set (n = 23), in which none of the samples had been used for its creation (Fig. 1). Interestingly, the ICPS accurately classified five out of six IC nonsurvivors and 16 out of 17 IC survivors (Figs. 5A and 5B, right), resulting in 91% (95% CI, 80%–100%) true predictive accuracy, 83% (95% CI, 68%–99%) sensitivity and 94% (95% CI, 84%–100%) specificity for poor-prognosis detection using a cutoff of −1.67 (Table IV). ROC plots of the ICPS showed that the clinical outcome could be predicted in this cohort with good true discriminatory ability (ROC area, 0.96; 95% CI, 0.88–1.00; p = 0.001; Fig. 5C, right).

With the purpose of strengthening the clinical performance of our ICPS, random selections of predictor variables (of comparable sizes) were also evaluated for class prediction accuracy of the test set using models built as outlined above. Not surprisingly, molecular fingerprints of these random combinations yielded poor true predictive accuracies to classify survivors and nonsurvivors in the test set (c-statistic, 0.51–0.67; p = 0.2–0.9).

Prognostic Power of the ICPS—We next investigated the ICPS ability to predict the risk of a fatal outcome in IC patients within 2 months following presentation (Fig. 1). Univariate logistic-regression models indicated that each unit increase in our ICPS, random selections of predictor variables (of comparable sizes) were also evaluated for class prediction accuracy of the test set using models built as outlined above. Not surprisingly, molecular fingerprints of these random combinations yielded poor true predictive accuracies to classify survivors and nonsurvivors in the test set (c-statistic, 0.51–0.67; p = 0.2–0.9).

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A 5-IgG Antibody-Reactivity Signature for IC Prognosis

Fig. 6A). These differences were more prominent when the test cohort was dichotomized according to the ICPS cutoff defined in the training set. IC patients with an ICPS $> -1.67$ (low-risk group) had a 99% (95% CI, 76%–100%) less mortality risk than those with an ICPS $\leq -1.67$ (high-risk group) ($p = 0.004$). Adjustment for known prognostic factors for IC and other baseline variables (potential confounders) did not significantly weaken the strong prognostic value of the ICPS in the test set (multivariate-adjusted OR, 0.04–0.57; $p = 0.05$).

After validating its prognostic power, the IPCS was applied to the entire-data set ($n = 45$) in an effort to more reliably examine the effect size. Similar risk patterns were evidenced when all 45 patients were considered together. The Hosmer-Lemeshow test demonstrated good fit to data of all these multivariate models ($p = 0.7–1.0$), confirming their validity.

To further visualize the relationship between established IC prognostic factors and the ICPS, clustering analyses were then conducted in the test set. PCA revealed that conventional prognostic factors for IC clustered into two groups, based on their association with the immune system status of IC patients, and away from the ICPS (Fig. 6C). HCA substantiated these results, showing no connection of the ICPS with specific branches (Fig. 6C).

Comparison of the ICPS and Traditional IC Prognostic Factors—To determine whether the ICPS added prognostic information beyond that provided by classical IC prognostic factors, we next created further multivariate models based on them (Table I) without and with the ICPS (referred to as traditional prognostic factors-index (TPFI) and TPFI + ICPS, respectively) in the same way as detailed above, and compared our ICPS with these models in the test cohort (Fig. 1). The ICPS was a stronger IC prognostic predictor than the TPFI (OR, 0.48 versus 0.74; $p = 0.008$ versus 0.05; Fig. 7A). Clinical concordance in outcome prediction for individual IC patients proved moderate-to-good between ICPS and TPFI + ICPS ($k = 0.5–0.8$; $p < 0.02$; Fig. 7B). Although the TPFI (ROC area, 0.79; 95% CI, 0.59–0.99; $p = 0.04$) and the
ICPS used both alone (ROC area, 0.96; 95% CI, 0.88–1.03; \( p = 0.001 \)) and in combination (ROC area, 0.94; 95% CI, 0.84–1.04; \( p = 0.002 \)) were able to discriminate between survivors and nonsurvivors (Fig. 7C), differences in their ROC areas revealed that the ICPS was more accurate in predicting clinical outcomes in IC than the TPFI (\( Z = 2.00; \) ...
p = 0.04; Fig. 7C, left) or individual classical IC prognostic factors ($Z > 1.96; p < 0.05$), and that the combined model (TPFI+ICPS) did not contribute to a better discrimination beyond the ICPS ($Z = 0.29; p = 0.8$; Fig. 7C, right).

Development of a Multiplexed Immunoassay for the Five Signature IgG Antibodies—To further validate the prediction model derived from SERPA, we next developed a multiplexed dot-blot assay for simultaneous and rapid measurement of the five signature IgG antibodies in each serum specimen (Fig. 1). First, Met6p, Ssb1p, Gap1p/Tdh3p, Hsp90p, and Pgk1p were expressed in E. coli as GST-fusion proteins, purified by affinity chromatography, and treated with a protease to remove the GST moiety (Fig. 8A). Their identity was then verified by MS analysis (Supplemental Fig. S2), and retention of their antigenic properties was confirmed by Western-blotting using three IC samples with different serum levels of the five signature IgG antibodies.

After empirically optimizing the best conditions for the multiplexed immunoassay, we evaluated its analytical performance (Figs. 8B–8E). Calibration curves for each signature IgG anti-
Table IV

Test operating characteristics of the ICPS in the LOOCV training, test and entire-data sets

| Test operating characteristics of the ICPS<sup>a</sup> | Mortality predictor for IC (≤ −1.67)<sup>b</sup> | Survival predictor for IC (>−1.67)<sup>b</sup> |
|--------------------------------------------------|---------------------------------|---------------------------------|
|                                                  | LOOCV training set (n = 22)     | LOOCV training set (n = 22)     |
|                                                  | Test set (n = 23)               | Test set (n = 23)               |
|                                                  | Entire-data set<sup>c</sup> (n = 45) | Entire-data set<sup>c</sup> (n = 45) |
| Percentage (95% CI)                              |                                 |                                 |
| Sensitivity                                      | 83 (68–99)                      | 100                             |
| Specificity                                      | 100                             | 83 (68–95)                      |
| Positive predictive value                        | 100                             | 94 (84–100)                     |
| Negative predictive value                        | 94 (84–100)                     | 94 (84–100)                     |
| Accuracy                                         | 95 (84–100)                     | 95 (84–100)                     |
| Classification error                             | 5 (3–7)                         | 5 (3–7)                         |
| Ratio (95% CI)                                   |                                 |                                 |
| Positive likelihood ratio                        | 14.17 (0.00–28.42)              | 6.00 (3.95–8.05)                |
| Negative likelihood ratio                        | 0.17 (0.01–0.32)                | 0.07 (0.05–0.09)                |
| Prognostic odds ratio                            | 80.00 (63.65–96.35)<sup>d</sup> | 80.00 (63.65–96.35)<sup>d</sup> |
| Mean±S.E. (95% CI)                               | 0.97 ± 0.04 (0.90–1.00)<sup>f</sup> | 0.96 ± 0.04 (0.88–1.00)<sup>f</sup> |

- <sup>a</sup> The ICPS is a simplified clinical-outcome prediction model for IC based on serum five-IgG antibody-reactivity signatures in IC patients at presentation (see Fig. 4). The test operating characteristics summarized in the table are for the ICPS modeled as a mortality or survival predictor for IC in order to identify IC patients who had a poor or good clinical outcome, respectively, in the ensuing 2-month period after presentation.
- <sup>b</sup> Model cutoffs were defined on the basis of ROC curve analysis on training set data (see Fig. 4D). These discrimination threshold values correspond to the highest combined sensitivity and specificity (i.e., the minimum sum of false-negative plus false-positive test results) for poor or good clinical outcomes when mortality or survival predictors, respectively, for IC were determined.
- <sup>c</sup> To minimize the likely overestimation of the prognostic value of the ICPS, data derived both from LOOCV training set rather than original training set and from the test set were considered in the entire data set (see Fig. 5C).
- <sup>d</sup> The odds for a positive test result in IC patients who died within 2 months relative to the odds of a positive result in those who had a favorable clinical outcome.
- <sup>e</sup> The odds for a positive test result in IC patients who had a favorable clinical outcome relative to the odds of a positive result in those who died within 2 months.
- <sup>f</sup> p < 0.001 as compared with an area of 0.5 for a nonuseful test. A value ranging from 0.7 to 0.8 indicates reasonable discrimination, and a value more than 0.8 represents good discrimination (51).
- <sup>g</sup> p < 0.001 as compared with an area of 0.5 for a nonuseful test. See footnote <sup>f</sup>.
body resulted in a good log-linear response within working range, in the interval of 0.5–20 RU/ml (R² = 0.988–0.995; Fig. 8F). Limit of detection ranged from 0.08 (Met6p-IgG) to 0.21 (Gap1p/Tdh3p-IgG) RU/ml (Fig. 8F), and limit of quantification ranged from 0.22 (Met6p-IgG) to 0.38 (Ssb1p-IgG) RU/ml (Fig. 8G). The multiplexed immunoassay was reproducible in the entire range of analyzed concentrations (Fig. 8G) and, overall, it showed good intra- and interassay imprecision for each signature IgG antibody (Supplemental Table S2). The assay achieved good dilution linearity, with a median dilution recovery of 103% (range, 81%–118%; Fig. 8H). Analytical recovery of exogenously added signature IgG antibodies was almost complete (median, 97%; range, 80%–117%; Fig. 8I). Dilution/analytical recovered and expected concentrations of each signature antibody correlated well (r = 0.996 for dilution linearity, and 0.982 for analytical recovery; p < 0.001 for both analyses) and did not significantly differ (p = 0.6 and 0.7 for linearity and recovery studies, respectively). Accordingly, sample dilution did not systematically affect the multiplexed immunoassay nor did potential interfering substances present in the sample matrix (Figs. 8H, 8I).
Having proven that our multiplexed immunoassay fulfilled the requirements of detection limit, functional sensitivity, imprecision, linearity and analytical recovery, we next assessed its prognostic performance in the test cohort (Fig. 9A). Unsupervised PCA separated testing IC survivors
and nonsurvivors in two distinct groups on the basis of their five-IgG antibody-reactivity profiles (Fig. 9B). As expected, not only did unsupervised HCA confirm these results (Fig. 9C) but it also revealed that the Met6p-Hsp90p-Pgk1p-IgG and Ssb1p-Gap1p/Tdh3p-IgG antibody-reactivity signatures were synergistic in predicting clinical outcomes in IC patients (Fig. 9D). Remarkably, no significant differences in known IC prognostic indicators and other baseline characteristics were identified between these antibody-reactivity subgroups.

These profiles were then modeled as described above. Supervised stepwise multivariate discriminant analyses with LOOCV identified the five signature IgG antibodies as the most simplified and accurate molecular fingerprint for clinical-outcome prediction in IC, from which a similar algorithm (IC multiplex antibody prognosis score or IC-MAPS) was derived. Low IC-MAPS values were associated with an adverse clinical outcome in IC patients (Figs. 9D–9F). Using a threshold of −0.97, the IC-MAPS properly classified 21 out of the 23 IC patients from the test set (Figs. 9E and 9F), resulting in the same accuracy (91%; 95% CI, 80%–100%) as the ICPS, 100% sensitivity, and 88% (95% CI, 75%–100%) specificity for poor-prognosis detection in IC. The IC-MAPS (ROC area, 0.98; 95% CI, 0.93–1.00; p = 0.001) showed a higher ability to discriminate IC survivors from non-IC survivors than individual signature IgG antibodies (Z = 2.04–2.30; p = 0.04–0.02; Fig. 9G), and it was not gained confidence when less than five signature IgG antibodies were tested. Its combination with the TPFI or individual classical IC prognostic factors did not lead to additionally improved clinical-outcome prediction for IC either.

Biologic Insights into the ICPS and Global Prognostic Antibody Signature for IC—In an endeavor to gain further insights into the biologic significance of the ICPS and molecular basis underlying the distinct clinical courses in IC, GO analyses were then performed (Fig. 1). Significant enrichments for host-interaction, protein-binding, steroid hormone signaling pathway, response to stress and glycolysis/glucogenesisis ontologies (for ICPS and global prognostic antibody signature) and entry into host, ATP binding and lyase activity ontologies (for global prognostic antibody signature) were identified in the good-prognosis signatures, whereas apoptosis and oxygen/reactive-oxygen-species metabolic process ontologies (for ICPS and global prognostic antibody signature) were over-represented in the poor-prognosis signatures.

**DISCUSSION**

**Differences in Anti-Candida IgG Antibody Reactivity Profiles among IC Patients at Presentation Reflect Distinct Clinical Outcomes**

We found that anti- *Candida* IgG antibody-reactivity patterns of IC patients at presentation were distinct according to their likely clinical outcomes within 2 months. This molecular heterogeneity may be ascribed to the expression of certain anti-Candida antibodies that can mediate protection against IC and consequently determine the IC fate (53–55). Accordingly, protective and nonprotective antibody responses, which appear to differ in epitope specificity, isotype, and/or titer (54, 55), could show separate effects to IC and delineate discrete molecular fingerprints corresponding to distinct patient outcomes. This may explain why the good-prognosis signature was characterized by higher levels of known pro-

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**Fig. 8. Analytical performance of the multiplexed immunoassay for simultaneous and rapid measurement of the five signature IgG antibodies in each serum sample.** A, Recombinant *C. albicans* proteins used as diagnostic reagents in the multiplexed immunoassay. Purified and Pre-Scission protease-treated recombinant Met6p (lane 1), Ssb1p (lane 2), Gap1p/Tdh3p (lane 3), Hsp90p (lane 4), and Pgk1p (lane 5) were separated on a 10% SDS-PAGE gel and silver-stained. Lane M, molecular mass standards. B, Representative immunorecognition patterns of threefold serial dilutions of the calibrator. In addition to the five recombinant *C. albicans* protein dots, each multiplexed immunoblot included negative (C−; bovine serum albumin) and positive (C+; *C. albicans* protein extract) control dots to monitor experimental performance. Numbers below each panel depict signature IgG antibody concentration. C, Typical multiplexed dot-blot array showing intra-assay imprecision of one serum with different signature IgG antibody concentrations in four replicates. See Supplemental Table S2 for further details. D, Representative multiplexed immunoblots depicting dilution effect (1:250–1:1000) in the assay buffer for one serum with different signature IgG antibody concentrations. E, Typical multiplexed dot-bots illustrating analytical recovery of known amounts of calibrator (0.5, 1.5, and 2.5 RU/ml signature IgG antibodies) added exogenously to one serum with different signature IgG antibody concentrations. F, Calibration curves for each signature IgG antibody. Threefold serial dilutions of a serum pool from one IC survivor and one IC nonsurvivor with an arbitrary value of 6000 RU/ml signature IgG antibodies were used as a calibrator, because no reference standards are available. The dose-response curves were fit to a four-parameter logistic-regression model. Each point represents the mean relative dot volume (for the relative concentration of the calibrator) of six different analytical assays, and bars designate standard error of the mean. Imprecision profiles for each signature IgG antibody. Inter-assay CVs at different concentrations of the calibrator were fit to a quadratic-regression model, and are given as the mean of different six determinations. The shaded rectangles show the portion of the curves with an optimal inter-assay imprecision. H, Dilution linearity for each signature IgG antibody. Six serum specimens with different signature IgG antibody concentrations were twofold serially diluted (from 1:250 to 1:1000) in the assay buffer. Dilution ratio and recovery are referred to the less diluted sample (1:250). I, Analytical recovery for each signature IgG antibody. Known amounts of calibrators (0.5, 1.5, and 2.5 RU/ml signature IgG antibodies) were added exogenously to six serum samples with different signature IgG antibody concentrations. In panels H and I, p values are for slope deviation from zero, each symbol designates an individual serum sample, and the numbers above or below each symbol depict the recovery percentage of signature IgG antibodies at that relative (H) dilution ratio or (I) concentration of calibrator added to the tested sample. For clarity, the scales on the y-axes of panels H and I are different. Square brackets in the axis legends denote concentration; AU, arbitrary units; RU, reference units; CV, coefficient of variation; LOD, limit of detection; LOQ, limit of quantification; S_pR2, standard deviation about the regression line; R2, goodness-of-fit statistic.
Fig. 9. Prognostic performance of the multiplexed immunoassay on the test cohort. A, Representative multiplexed dot-blot images showing serum specimens from seven and four IC patients with good and poor clinical outcomes, respectively, within 2 months following presentation and from one non-IC patient (negative control) with no detectable serum levels to the five signature IgG antibodies in a two-dimensional blot. In addition to the five recombinant C. albicans protein dots, each immunoarray included negative (C++; bovine serum albumin) and positive (C--; C. albicans protein extract) control dots to monitor experimental performance. Numbers above each pattern indicate the relative reactivity level of the antibody reactivity signature for IC prognosis.

The text continues with further analysis and discussion of the data presented in the figure.
tective antibodies, such as those to Hsp90p, Eno1p, Met6p, or Fba1p (13, 15, 56–62), whereas the poor-prognosis signature was associated with undetectable/lower levels of them (possibly insufficient to induce protection (55)) and higher levels of nonprotective antibodies, like those to Gap1p/Tdh3p (62, 63) or Ssa4p, Ssb1p or Ssc1p (cell wall-exposed, Hsp70p family members (42, 64)). The latter is gathered from the nonprotective effect of anti-surface Hsp70p antibodies (65) and their high homology degree (see below). Intriguingly, the presence of the 31 identified antigens at the Candida surface (42, 64) (probably solubilized in the whole soluble proteome given their loose association (64, 66, 67)) provides further support for our finding that generation of antibodies to some of them could be of benefit to the host and, feasibly, to future prophylactic/therapeutic purposes for IC.

The ICPS Derived from the Molecular Fingerprints of Five Anti-Candida IgG Antibodies May Reliably Predict the Clinical Outcome in Individual IC Patients at Presentation

Although our 31-IgG antibody-reactivity signature was able to discriminate between distinct clinical-outcome subgroups, this may be too complex and costly to apply as a clinical test in routine general practice. To reduce its dimension, we developed a classification algorithm that optimally combined the minimum number of predictor variables with the best accuracy. Because this type of nonbiological and mathematical multivariate classifiers is often associated with overfitting and potential bias or chance (52), we used cross-validation techniques to evaluate its internal consistency within a training set followed by further validation within an external test set. The similarity in clinical performances between the LOOCV training and test sets, poor predictive accuracies of molecular fingerprints of random combinations, and confirmation of SERPA-derived predictions by the multiplexed immunoassay validate our five-IgG antibody-reactivity signature as a reliable IC prognostic predictor. These findings are not only in line with an earlier report showing the SERPA utility for identifying individual IC prognostic candidates (anti-wall Eno1p or anti-Bgl2p IgG antibodies) (13) but also extend this potential to uncover a panel of optimally combined biomarkers that may cover a broader range of IC patients given host-response heterogeneity as well as novel therapeutic options against IC (see below). To our knowledge, this study is the first attempt to develop and validate a molecular predictor for IC prognosis.

We chose a multiplexed dot-blot assay for model validation because this is a simple, rapid, accurate and cost-effective method that can simultaneously measure several biomarkers in each sample. For small-scale validation studies, this format thus provides an attractive alternative both to the traditional ELISA, limited by its ability to quantify only a single biomaker in each sample, and to new high-throughput multiplexed technologies (such as multiplexed microplate or fluorescent beads-based immunoassays), which entail an elevated investment in equipment and disposable supplies (68). However, future large-scale validation studies will certainly require the latter given that these are easier-to-automate and will enable better data quantification. Clearly, our antibody-reactivity signature will have to be appropriately adjusted, optimized and further validated in these larger studies as these may, for example, lead to changes in the prognosis threshold value and/or relative contributions of the predictor variables in the multivariate model.

The ICPS Is Unbiased by and Outperforms Traditional IC Prognostic Factors

Because diverse patients-related variables represent another major source of potential bias or chance in these studies (38, 52), known IC prognostic factors and other baseline characteristics were assessed as potential confounders. Multiva-
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The ICPS May Be Useful for 2-Month Mortality Risk Level Estimation and More Appropriate Initial Therapeutic Decision-Making in Individual IC Patients

Another remarkable finding was the ICPS ability to properly discriminate IC patients at high-risk for death from those at low-risk. This could, if confirmed prospectively in a larger patient population, be valuable to adequately guide initial therapeutic decision-making in the future (1). This ICPS might therefore aid physicians in identifying IC patients at high-risk for a likely fatal outcome who may benefit from the best and most aggressive therapeutic method and closer clinical follow-up. Conversely, this molecular signature might also help them to select low-risk patients who may need less intensive clinical follow-up and/or be referred for healthcare strategies that aim to minimize antifungal therapy-related costs and (nephro)toxicities.

Antibody-Reactivity Profiling May Provide Biologic Insights into Molecular Mechanisms that Influence the Clinical Outcome in IC to Guide Future Hypothesis-Driven Studies

Beyond the practical impact of providing a prediction model for IC prognosis, SERPA-based profiling may also reveal dysregulated biological processes and/or molecular functions during distinct clinical outcomes in IC that could otherwise not be revealed. The noticeable enrichment for antibodies to pathogenicity-related proteins (involved in host protein binding, steroid hormone signaling pathway, stress response, and host tissue invasion (69–75)) in the good-prognosis signature may reflect important defense mechanisms responsible for Candida clearance. Neutralization of glycolysis and gluconeogenesis, ATP-binding, and lyase-activity proteins, prominent molecular features of the favorable clinical-outcome group, could represent other pathways that may also contribute to an overall improved prognosis. In fact, the breakdown of glycolysis and gluconeogenesis-related proteins has been associated with rapid or slow ATP depletion and subsequent cell necrosis or apoptosis, respectively (76). Mutations that eliminate nucleotide-exchange-factor activity of certain ATP-binding proteins have proved lethal (77). Although obvious link has been hitherto unreported between lyase activity and IC pathogenesis, this interaction may promote new research fields. All these processes/functions neutralized by the good-prognosis signature should be targeted as novel therapeutic options for IC in future studies. In contrast, apoptosis and reactive-oxygen-species metabolism neutralization were uncovered in the poor-prognosis signature as argued below.

The Biologic Relevance of the Antibodies Included in the ICPS to IC Prognosis Confirms Our Five-IgG Antibody Signature as a Credible Clinical-Outcome Predictor for IC

The Good Prognosis-Associated, Protective Antibodies in the ICPS are Directed to Putative Candida Virulence Factors and Antiapoptotic Mediators—Candida Met6p is essential for growth and viability, and is involved in virulence and morphogenesis (74, 78). Hsp90p is a putative Candida virulence factor (73, 79) that may interfere with proper human protein folding, functioning and/or degradation and activate human endothelial nitric oxide synthase (altering cardiovascular hemodynamics) (58, 80). Met6p and Hsp90p levels are increased in response to the human steroid hormone 17-β-estradiol (73, 74), which adds to

The Poor Prognosis-Associated, Risk Antibodies in the ICPS are Directed to Potential Candida Proapoptotic Mediators—Although the mechanisms by which antibodies to Gap1p/Tdh3p and Ssb1p may negatively affect the clinical outcome of IC patients are unclear, earlier studies strengthen our results (62, 63, 65, 87). Passive transfer and immunization assays in animal models have shown that anti-Gap1p/Tdh3p antibodies are neither protective nor can they stimulate protective responses against IC (62, 63). Likewise, not only does immunization with a surface-exposed Hsp70p induce nonpro
tective responses against IC, but it may also play an apparent role in accelerating the animal’s death (65). Clues as to these risk (nonprotective) patterns may come from the roles of Gap1p/Tdh3p and Hsp70p as proapoptotic mediators (88–90). Antibodies to Candida surface-associated Gap1p/Tdh3p (enzymatically active (91)) might either neutralize its activity and reduce the concentration of its intracellular form (a major messenger mediating apoptotic cell death (88, 92)) in compensatory response (oversecretion), or block it at the cell surface, where Gap1p/Tdh3p might act as an extracellular proapoptotic sensor. Both ways would lead to an increase in Candida survival and a poor prognosis in IC patients. Conversely, surface-associated Hsp70p can mediate apoptosis in tumor cells through specific binding and uptake of granzyme B released by natural killer cells, whose lytic activity can be inhibited by anti-surface Hsp70p antibodies (90, 93). Similarly, Candida surface-associated Ssa1p and Ssa2p (Hsp70p family members) mediate binding and uptake of human β-defensins 2/3 and histatin 5 required for their fungistic and fungicidal activity against Candida (94, 95). Both mechanisms could explain why anti-Ssb1p antibodies (which might analogously neutralize these or other innate immune system components) may not be of benefit to the host. Accordingly, our results might offer a basis for development of anti-idiotype antibodies, i.e. antibodies that block the antigen-combining (idiotypic) region of these risk antibodies, as novel IC therapeutics in the future.

**The Whole Five-IgG Antibody Set Appears to Have the Key Information for IC Prognosis**

High antibody levels to Ssb1p, Gap1p/Tdh3p and Pgk1p and low anti-Met6p antibody levels at early IC stages were associated with poor prognoses (15, 87). Likewise, a synthetic vaccine combining β-mannan and Pgk1p-derived peptide epitopes slightly enhanced IC susceptibility (62). This correlation between adverse outcomes and anti-Met6p or anti-Pgk1p antibody levels may result from the possibilities that these antibodies may not be present in adequate epitope specificity, isotype, and/or titer to induce protection (54, 55), and/or that whole set of reactivity profile of our five-IgG antibodies could have the key information for clinical-outcome prediction in IC. The latter is reinforced by the ICPS calculated for these published data (15, 87), which was predictive of a poor prognosis (2.78).

**Future Challenges**

Our study has revealed that prognostic antibody-reactivity signatures are present in IC patients at presentation and that these may be useful to formulate a molecular predictor of clinical outcome for IC and provide potential targets for upcoming molecular therapies against IC. However, before its routine implementation in clinical practice, our IC prognostic predictor must be refined and validated in future larger longitudinal prospective cohort studies, which are now ongoing, with adjustments for known IC prognostic factors and time-to-event analyses using easier-to-automate multiplexed clinical platforms. If confirmed, the resulting signature might open the way for individual prediction of clinical outcomes in IC.

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