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Predicting Invasive Aspergillosis in Hematology Patients by Combining Clinical and Genetic Risk Factors with Early Diagnostic Biomarkers

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ABSTRACT Personalized medicine provides a strategic approach to the management of IA. The incidence of IA in high-risk hematology populations is relatively low (<10%), despite unavoidable *Aspergillus* exposure in patients with a potentially similar clinical risk. Nonclinical variables, including genetic mutations that increase susceptibility to IA, could explain why only certain patients develop the disease. This study screened for mutations in 322 hematology patients classified according to IA status and developed a predictive model based on genetic risk, established clinical risk factors, and diagnostic biomarkers. Genetic markers were determined by real-time PCR and, with clinical risk factors and *Aspergillus* PCR results, subjected to multilogistic regression analysis to identify a best-fit model for predicting IA. The probability of IA was calculated, and an optimal threshold was determined. Mutations in dectin-1 (rs7309123) and DC-SIGN (rs11465384 and rs7248637), allogeneic stem cell transplantation, respiratory virus infection, and *Aspergillus* PCR positivity were all significant risk factors for developing IA and were combined in a predictive model. An optimal threshold requiring three positive factors generated a mean sensitivity/specificity of 70.4%/89.2% and a probability of developing IA of 56.7%. In patients with no risk factors, the probability of developing IA was 2.4%, compared to >79.1% in patients with four or more factors. Using a risk threshold of 50%, preemptive therapy would have been prescribed for 8.4% of the population. This pilot study shows that patients can be stratified according to risk of IA, providing personalized medicine based on strategic evidence for the management of IA. Further studies are required to confirm this approach.

KEYWORDS invasive aspergillosis, risk factors, SNPs, *Aspergillus* PCR, predictive model

The limited ability to accurately diagnose invasive aspergillosis (IA) has led to an overreliance on empirical antifungal therapy (1). In recent years, the incorporation of highly sensitive nonculture diagnostics (PCR, galactomannan [GM] enzyme immunoassay [EIA], and β-D-glucan detection) has increased diagnostic accuracy, enabling disease to be excluded and decreasing unnecessary antifungal use (2–4). Early diagnosis is important for good prognosis, and preemptive approaches utilizing nonculture-based tests can provide early evidence of infection (2–5). However, biomarkers alone are an insufficient reason to initiate therapy, as false-positive results occur. Biomarkers are best used to exclude disease when results are negative, with positive results used to trigger further clinical investigations (e.g., bronchoscopy and high-resolution computed tomography [HRCT]) to confirm disease.

A preemptive strategy of managing IA may have advantages. By definition, a preemptive approach involves taking action against an anticipated outcome, dependent on the likelihood of the patient developing disease in the future as determined

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by risk factors. While this is similar to a prophylactic strategy, it differs because of the availability of sensitive biomarker assays and the necessity for biomarker positivity during the early infective processes prior to clinically overt disease. The threshold at which preemptive action is taken is critical; if it is too low, the number of patients treated may be greater than that produced by comparable empirical policies, and if it is too stringent, the opportunity to prevent disease is missed. Thresholds can be dynamic, dependent on perceived and potential outcomes, which may also alter the risk.

There are multiple risk factors for IA in the hematology patient population (6–8). Many are associated with the underlying hematological malignancy and treatment, including prolonged neutropenia, lymphopenia, allogeneic stem cell transplantation (SCT), iron overload, graft-versus-host disease (GVHD), prolonged corticosteroid use, and monoclonal antibody use. Infection with other microbes (cytomegalovirus [CMV] and/or respiratory viruses), can also increase the risk of IA. Exposure to *Aspergillus* is unavoidable, but exposure to high levels of the organism during building construction or housing in contaminated environments increases the risk of disease.

Recently, genetic predisposition has been recognized as a risk factor for fungal infection. Research has focused on single nucleotide polymorphisms (SNPs) in genes coding for proteins involved in innate and adaptive immune responses. C-type lectin receptors (dectin-1, dectin-2, mannose binding lectin, DC-SIGN, and mincle) play a primary role in fungal immunity, and much research has concentrated on these targets (9). Mutations in other pattern recognition receptors (Toll-like receptors) have also been associated with an increased risk of IA (10). These genetic risk factors, although relatively nonspecific, are present before infection and provide an opportunity to stratify patients according to risk. Risk factor stratification may improve patient management but requires a combination of host, clinical, and early diagnostic markers.

Non-culture-based diagnostic assays can detect early infective processes, and the aim is to detect infection before overt tissue damage occurs. GM is mainly released from actively growing hyphae, and its presence in the circulation indicates hyphal growth and invasion (11). *Aspergillus* PCR has been shown to be positive earlier than GM and (1-3)-β-D-glucan (2, 12, 13). In an animal model of IA, blood was *Aspergillus* PCR positive on a time scale that related to exposure rather than disease (13). While this may reduce PCR specificity, it may be more beneficial in preemptive roles in patients stratified according to risk.

The aim of this study was not to identify novel clinical and genetic risk factors for IA but rather to combine (i) risk factors previously associated with the disease with (ii) a well-validated *Aspergillus* PCR assay standardized in accordance with international recommendations into a predictive model to determine the probability of developing IA. Genetic markers in dectin-1 and DC-SIGN previously associated with IA were retrospectively combined with established clinical risk factors and *Aspergillus* PCR screening results in a cohort of high-risk patient to determine whether a management strategy could stratify patients according to risk, providing personalized medical care to preempt disease (14).

MATERIALS AND METHODS

Patient population and study design. As part of the local neutropenic fever care pathway, hematology patients are routinely monitored for invasive fungal disease (IFD) by PCR and GM EIA (2, 15). Patients were included on the certainty of diagnosis assigned by the EORT-MSG criteria (16). If patients were defined as having proven, probable, or possible IA, they were included as cases. If patients had absolutely no radiological or mycological evidence of IA, they were included as controls. Subsequently, patients with nonspecific radiology and mycological evidence were excluded to maintain definitive case-control categorization. Retrospectively, a total of 322 hematology patients were anonymized and stratified according to IA diagnosis by using the revised European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG) definitions (16). There were 6 proven IA, 48 probable IA, and 20 possible IA patients and 268 patients with no evidence of fungal disease (NEF). Given the lower certainty of diagnosis, cases of possible IA were excluded from statistical analysis. Patients were treated for malignancy and other hematological/autoimmune conditions between October 2005 and June 2009. Known risk factors for developing IA were linked to the disease (Table 1.). As all patients are

TABLE 1 Patient demographics, underlying diseases, hematopoietic SCT status, biomarker (PCR) positivity, and additional viral infections stratified by IA classification

Parameter	Proven IA (n = 6)	Probable IA (n = 48)	Possible IA (n = 20)	NEF (n = 268)	P value[f]
Male/female ratio	2:1	1.7:1	1.9:1	1.5:1	0.7667
Median age (yr)	58	52	64	61	0.31[g]
No. with underlying disease					
AL/MDS	3	25	15	91	**0.011**
Lymphoma	1	13	3	90	0.3389
Myeloma	1	4	0	50	0.1134
Chronic leukemia	1	4	2	18	0.5605
Other[a]	0	2	0	19	0.5521
Mortality rate (%)	66.7	54.2	60.0	45.5	0.1302
Transplant rate (%)					
Combined	50.0	58.3	20.0	33.6	**0.0018**
Allogeneic SCT	50.0	45.8	20.0	9.3	**<0.0001**[h]
Autologous	0.0	12.5	0.0	24.3	**0.00321**
No transplant	50.0	41.7	80.0	66.4	**0.0018**
No. with GVHD	2	12	2	9	0.2757[i]
No. PCR positive					
1 positive threshold[b]	6	44	15	93	**<0.0001**
≥2 positive threshold[c]	6	36	10	39	**<0.0001**
No. with additional infection(s)					
CMV	4	11	3	22	0.3494[j]
Respiratory virus	1	34	4	38	**0.0025**[k]
Influenza A or B virus	0	9	1	11	0.2113[k]
Parainfluenza	1	7	1	8	0.0988[k]
Rhinovirus	0	5	0	10	0.4879[k]
RSV	0	12	2	9	**0.0046**[k]
Adenovirus	0	1	0	0	0.2996[k]
Multiple respiratory viruses	0	8[d]	0	8[e]	0.1483[k]

[a]Includes eight cases of aplastic anemia, three cases of myelofibrosis, three cases of unspecified leukemia, two cases of paroxysmal nocturnal hemoglobinuria, two cases of unspecified hematological malignancy, one case of amyloidosis, one case of an unspecified autoimmune disorder, one case of IgM paraproteinemia, and one case of posttransplant lymphoproliferative disorder.

[b]A single positive PCR result is considered significant.

[c]Multiple (two or more) positive PCR results are required to be considered significant.

[d]Includes 20 infections in eight cases of proven/probable IA. Multiple infections include two cases of influenza/parainfluenza virus infection, one case of adenovirus/rhinovirus infection, one case of influenza/parainfluenza/RSV/rhinovirus infection, one case of influenza/RSV/rhinovirus infection, one case of influenza/rhinovirus infection, one case of parainfluenza/rhinovirus infection, and one case of influenza/parainfluenza/rhinovirus infection. These multiple infections are included in the individual breakdown of respiratory virus infections.

[e]Includes 18 infections in eight cases with no evidence of IFD. Multiple infections include four cases of parainfluenza/rhinovirus infection, one case of influenza/parainfluenza/RSV infection, one case of influenza/RSV/rhinovirus infection, one case of influenza/parainfluenza virus infection, and one case of RSV/rhinovirus infection. These multiple infections are included in the individual breakdown of respiratory virus infections.

[f]Significance was adjusted to 0.0125 by the Bonferroni adjustment method to account for the presence of four potential risk factors (underlying disease, SCT, GVHD, and viral infection). P values indicating significant differences between proven/probable IA and patients with NEF are in bold.

[g]Adjusted to account for association with allogeneic SCT, GVHD, and respiratory virus status.

[h]Adjusted to account for associations with GVHD, CMV, and respiratory virus status.

[i]Adjusted to account for associations with allogeneic SCT, CMV, and respiratory virus status.

[j]Adjusted to account for association with allogeneic SCT, GVHD, and respiratory virus status.

[k]Adjusted to account for association with allogeneic SCT, GVHD, and CMV status.

screened as part of the neutropenic fever care pathway, neutropenia and fever were not included as risk factors. Information on underlying disease, clinical course (SCT, GVHD, and other infections), radiological history, results of all IA biomarkers, and mortality rates was gathered as part of the routine clinical management. No additional information outside this remit was sought. Clinical features (histology/HRCT) or mycological (GM EIA/culture) evidence used to define disease within consensus criteria were not included as risk factors to avoid incorporation bias.

The presence of SNPs previously associated with an increased risk of IA was determined (14). SNPs were chosen on the basis of their availability as commercially available TaqMan assays, providing methodological simplicity and standardization and quality control. The SNP assays were retrospectively performed with stored genomic DNA previously extracted as a by-product of *Aspergillus* PCR screening. No additional samples specific for this purpose were requested, and the local ethics board ruled that the project did not require ethical approval and was approved by the research-and-development board.

GM EIA. Testing by Platelia *Aspergillus* EIA (Bio-Rad) was undertaken in accordance with the manufacturer's instructions. Optical densities (OD) at 450 and 620 nm were read (Thermo Scientific

TABLE 2 Prevalence of SNPs associated with IA in 54 hematology patients with EORTC-MSG-defined proven/probable IA and 268 patients with NEF[a]

Gene product	Locus	ABI assay	No. with proven or probable IA/total	No. with NEF/total	OR (95% CI)	*P* value[b]
Dectin-1	rs16910526	C_33748481_10	12/54	51/268	1.2 (0.6–2.6)	0.576
Dectin-1	rs7309123	C_3130832_10	47/54	173/268	3.7 (1.5–9.3)	**0.001**
DC-SIGN	rs11465384	C_25996399_10	15/54	30/268	3.1 (1.4–6.5)	**0.004**
DC-SIGN	rs7252229	C_29620333_10	17/54	55/268	1.8 (0.9–3.6)	0.106
DC-SIGN	rs7248637	C_29710787_10	18/54	37/268	2.9 (1.4–5.9)[a]	**0.001**[c]
Combination	Haplotype and rs7309123	C_3130832_10, C_25996399_10, C_29710787_10	20/54	31/268	4.5 (2.2–9.2)	**<0.001**

[a]The prevalence of the SNP is based on a combined homozygous-heterozygous allele rate.
[b]*P* values indicating significant associations are in bold. Significance was adjusted to $P = 0.01$ by the Bonferroni adjustment method.
[c]Adjusted for significant associations with AL/MDS.

Multiskan FC). Indices were calculated by dividing the OD of the sample by the mean OD of two threshold controls included in the kit. All samples were considered positive if the sample index was ≥0.5.

***Aspergillus* PCR testing.** Molecular testing was performed as previously described, by using well-established methods that were compliant with international recommendations (2, 15, 17). In brief, DNA was extracted from a minimum of 3 ml of EDTA whole blood subjected to red and white cell lysis prior to bead beating and subjected to automated DNA purification/precipitation with the Qiagen EZ1 system. Both human DNA and *Aspergillus* DNA, when present, were coextracted. *Aspergillus* PCR targeted the 28S rRNA gene with a limit of detection of 3 input copies per reaction, and an internal control was used to monitor for inhibition. The clinical performance of the PCR has been previously described (15, 18).

Determination of genetic risk factors. Commercial TaqMan SNP assays (Applied Biosystems) for a total of five SNPs previously associated with IA were available (Table 2) (14, 19). Genomic DNA was extracted as a by-product of routine *Aspergillus* PCR testing as described above. Prior to testing, the concentration and quality (A_{260}/A_{280} ratio) of the extracted DNA were determined with a P-300 nano-photometer (Implen, Germany). Genotyping was performed in accordance with the manufacturer's recommendations with an ABI 7500 Fast instrument.

Each sample was anonymously tested in duplicate when sufficient DNA was available. For patients undergoing allogeneic SCT, one sample was tested prior to SCT and one was tested several weeks after SCT. If discordant results were obtained, additional samples were tested before and after SCT to confirm a genotypic switch after SCT.

Statistical analysis. Genotype and clinical risk factor frequencies in the proven/probable IA and NEF groups were compared by using the Fisher exact test and the Pearson χ^2 test. Odds ratios (OR) and 95% confidence intervals (CI) were calculated for the presence (combined homozygous and heterozygous SNPs) or absence (homozygous wild-type allele) of the polymorphisms and clinical risk factors. The thresholds for the significance of clinical and genetic risk factors were separately adjusted for multiple variables by the Bonferroni adjustment method (clinical factor significance, $P = 0.0125$; genetic factor significance, $P = 0.01$). Pairwise logistic regression was used to identify any associations between independently significant risk factors, and when necessary, adjustments were performed by Mantel-Haenszel OR and a χ^2 test of heterogeneity was used to determine the significance of the adjustment. For each SNP, consistency with the Hardy-Weinberg equilibrium was determined by a standard observed-expected χ^2 test with a *P* value at 1 degree of freedom. To determine any genetic linkage between significant SNPs, linkage disequilibrium (LD) was calculated and an exclusion threshold was set at an r^2 value of ≥0.8. To provide clinical utility, the probability of IA associated with a risk factor was also calculated. Receiver operating characteristic (ROC) analysis was performed to determine an optimal threshold for the final model.

RESULTS

Genetic markers and IA. Three SNPs (dectin-1 rs7309123 and DC-SIGN rs11465384 and rs7248637) showed a significant correlation with proven/probable IA (Table 2). All five SNPs were consistent with Hardy-Weinberg equilibrium. LD analysis showed that SNPs rs11465384 and rs7248637 were in LD with each other ($r^2 = 0.6$) but did not reach the prerequisite threshold for exclusion (an r^2 value of ≥0.8).

Given the association between DC-SIGN rs11465384 and rs7248637 (OR, 54.3; 95% CI, 22.8 to 129.2; $P < 0.0001$), it was decided to consider them a haplotype and combine data for further analysis; the association with IA remained significant (OR, 3.0; 95% CI, 1.6 to 5.7; $P = 0.0011$). Logistic regression identified only limited associations between clinical risk factors and genetic markers, and the data were adjusted accordingly (Table 2). There were no associations between any of the SNPs and other infections (CMV, respiratory virus) included in this study.

As individual assays, none of the significant SNPs could be used to confidently diagnose IA (highest positive likelihood ratio [LR], 2.48) or exclude IA (lowest negative

TABLE 3 Probability of IA in the presence of clinical conditions and risk factors

Risk factor(s)	% of population (n = 322)	OR (95% CI)	P value
AL/MDS[a]	37.0	2.1 (1.1–3.9)	0.011
AL/MDS − allogeneic SCT + respiratory virus[b]	4.3	4.2 (1.0–17.7)	0.036
AL/MDS + allogeneic SCT[c]	6.2	10.4 (3.2–35.1)	<0.001
AL/MDS + allogeneic SCT + respiratory virus[d]	2.8	14.4 (1.1–412.5)	0.029
Other[e]	63.0	0.5 (0.3–0.9)	0.011
Other − allogeneic SCT + respiratory virus[f]	4.7	3.3 (0.6–15.7)	0.107
Other + allogeneic SCT[g]	9.3	7.6 (2.8–20.8)	<0.001
Other + allogeneic SCT + respiratory virus[h]	3.7	4.0 (0.6–31.6)	0.127

[a]Population used for analysis of AL/MDS (n = 119, 28 with IA) versus other hematological malignancy (n = 203, 26 with IA).
[b]Population used for analysis of AL/MDS without allogeneic SCT but positive for respiratory virus infection (n = 14, 5 with IA) versus AL/MDS without allogeneic SCT and no evidence of respiratory virus infection (n = 85, 10 with IA).
[c]Population used for analysis of AL/MDS with allogeneic SCT (n = 20, 13 with IA) versus AL/MDS without allogeneic SCT (n = 99, 15 with IA).
[d]Population used for analysis of AL/MDS with allogeneic SCT and respiratory virus infection (n = 9, 8 with IA) versus AL/MDS without allogeneic SCT but with respiratory infection (n = 14, 5 with IA).
[e]Population used for analysis of other hematological malignancy (n = 203, 26 with IA) versus AL/MDS (n = 119, 28 with IA). Other includes lymphoma, myeloma, chronic leukemia, aplastic anemia, myelofibrosis, unspecified leukemia, paroxysmal nocturnal hemoglobinuria, unspecified hematological malignancy, amyloidosis, an unspecified autoimmune disorder, IgM paraproteinemia, and posttransplant lymphoproliferative disorder.
[f]Population used for analysis of other hematological malignancy without allogeneic SCT but positive for respiratory virus infection (n = 15, 3 with IA) versus other hematological malignancy without allogeneic SCT and no evidence of respiratory virus infection (n = 158, 11 with IA).
[g]Population used for analysis of other hematological malignancy with allogeneic SCT (n = 30, 12 with IA) versus other hematological malignancy without allogeneic SCT (n = 159, 14 with IA).
[h]Population used for analysis of other hematological malignancy with allogeneic SCT and respiratory virus infection (n = 12, 6 with IA) versus other hematological malignancy without allogeneic SCT but with respiratory infection (n = 15, 3 with IA).

LR, 0.37). Even if all three significant SNPs were present in an individual (9.0% of the population), the positive LR for developing IA was only 4.0, and if they were negative for all significant SNPs (27.6% of the population), the negative LR was 0.2.

A multivariate regression model (model fit P = 0.0003) of genetic markers factors confirmed significant associations between the DC-SIGN haplotype (P = 0.0126) and dectin-1 rs7309123 (P = 0.0035) and the development of IA. These were retained for inclusion in the final model.

Clinical risk factors for IA. The analysis of this population confirmed previous findings. Significant associations were found between IA and acute leukemia/myelodysplastic syndrome (AL/MDS; OR, 2.095; 95% CI, 1.115 to 3.941), use of allogeneic SCT (OR, 2.90; 95% CI, 1.25 to 6.69), and infections with respiratory viruses (OR, 3.22; 95% CI, 1.44 to 7.21), mainly driven by respiratory syncytial virus (RSV) infection (OR, 3.47; 95% CI, 1.38 to 8.69) (Table 1).

Table 1 shows that there was a difference in age between patients with proven/probable IA and those with NEF, but upon adjustment for allogeneic SCT, it was not significant. The same was true of CMV infection and GVHD, where the initial analysis showed a significant association with IA (CMV OR, 4.301; 95% CI, 1.929 to 9.561; P <0.0001; GVHD OR, 10.07; 95% CI, 3.78 to 27.3; P <0.0001), but this was removed after adjustment for SCT. Respiratory virus infections retained a significant independent association with IA even after adjustment (Table 1).

A multivariate regression model (model fit P < 0.0001) of clinical factors confirmed significant associations between the development of IA and a diagnosis of AL/MDS (P = 0.0314), allogeneic SCT (P = 0.0021), and respiratory virus infection (P = 0.0043). The odds of developing IA in the presence of the various significant clinical factors are shown in Table 3. For the AL/MDS population, allogeneic SCT and respiratory virus infection were again significantly associated with IA. For the non-AL/MDS population, allogeneic SCT remained significant whereas respiratory virus infection was not significant, albeit the numbers were limited. The variables underlying hematological disease, allogeneic SCT, and respiratory virus infection were retained for inclusion in the final model.

***Aspergillus* PCR.** The sensitivity, specificity, positive LR, negative LR, and diagnostic OR for *Aspergillus* PCR using a single positive result as significant were 92.6% (95% CI,

82.0 to 97.6), 65.3% (95% CI, 63.2 to 66.3), 2.7, 0.1, and 27, respectively. Although there was a strong association between PCR result and IA status (OR, 23.5; 95% CI, 7.8 to 79.2; $P < 0.0001$), PCR can be used to confidently exclude IA but diagnosis is hampered by false positivity. Using a multiple-positive-PCR threshold significantly increased specificity (85.4%; 95% CI, 83.0 to 87.3), although it still could not be used solely to confirm a diagnosis of IA (positive LR, 5.3). *Aspergillus* PCR testing was retained for inclusion in the final model.

Combined prediction model. In determining the final model, clinical risk factors (underlying hematological malignancy [AL/MDS], allogeneic SCT, respiratory virus infection), the presence of significant genetic markers (DC-SIGN haplotype and dectin-1 rs7309123), and mycological evidence not used to categorize IA disease (*Aspergillus* PCR result) were combined.

On combination of these variables, underlying hematological malignancy was no longer significantly associated with IA ($P > 0.5772$) and was removed from the model. The final model fit was $\chi^2 = 106.4$ ($P < 0.0001$). The probability of developing IA associated with various combinations of clinical risk factors, genetic markers, and biomarker positivity is shown in Fig. 1A and B. For patients not receiving, or prior to, allogeneic SCT, the risk of developing IA, even in the presence of genetic markers, was low (<5%). Consequently, 59.6% (162/272) of the patients without allogeneic SCT could be considered at low risk and only patients with multiple genetic markers, who were *Aspergillus* PCR positive and had a respiratory virus infection (1.8% [5/272] of this population), had a risk of IA that exceeded 50% (Fig. 1A and 2).

The majority (96%) of patients after allogeneic SCT had a greater risk of IA (>5%), with a significantly higher risk (>50%) for 44% (22/50) for allogeneic SCT patients who were *Aspergillus* PCR positive and had a respiratory infection or who had multiple genetic markers and were either *Aspergillus* PCR positive or had a respiratory infection (Fig. 1B and 2). The risk of IA for 10% (5/50) of the allogeneic SCT patients with multiple genetic markers who were PCR positive and had respiratory virus infection was 89.9%.

When this model is applied to the entire hematology population ($n = 322$), 50.9% are considered to have a low risk (<5%) and 8.4% are considered to have a high risk (>50%) of developing IA. A risk breakdown is shown in Fig. 2.

ROC analysis of the final model generated an area under the curve of 0.8633 (Fig. 3). If one variable was positive, the sensitivity was 98.2% (95% CI, 90.1 to 99.9%) and the negative LR was 0.12, whereas if four or more variables were positive, the specificity was >98.15% (95% CI, 95.7 to 99.4%) and the positive LR was >18.8. The mean probability that a patient with four risk factors would develop IA was 79.2%. The optimal threshold to start preemptive therapy required three variables to be positive, and the sensitivity and specificity were 70.4% (95% CI, 56.4 to 82.0%) and 89.2% (95% CI, 84.8 to 92.6%). The corresponding positive and negative LRs were 6.50 and 0.332, and in patients with three risk factors, the mean probability of developing IA was 56.7%, compared to 6.3% in patients with fewer than three risk factors. The number of risk factors needed to justify treatment was 1.69 (95% CI, 1.6 to 2.6). Using a threshold of two risk factors positive, the sensitivity and specificity of the model were 90.7% (95% CI, 79.7 to 96.9%) and 62.3% (95% CI, 56.2 to 68.1%) and the positive and negative LRs were 2.41 and 0.149, respectively. The mean probability of developing IA for patients with two or more risk factors was 32.6%, compared to 2.9% for patients with fewer than two risk factors.

DISCUSSION

The purpose of this study was to develop a model combining genetic markers with established clinical risk factors and biomarker screening to stratify hematology patients at risk of developing IA. By linking the high sensitivity of PCR testing (sensitivity, 92.6%; negative LR, 0.11) with specificity driven by combined clinical factors and genetic susceptibility (pairwise specificity range, 94.0 to 99.6; positive LR, 6.2 to 37) (Fig. 1A and B), diagnostic performance is improved. ROC analysis showed that the model was able to both exclude and diagnose IA (Fig. 3). Using the optimal threshold of three positive

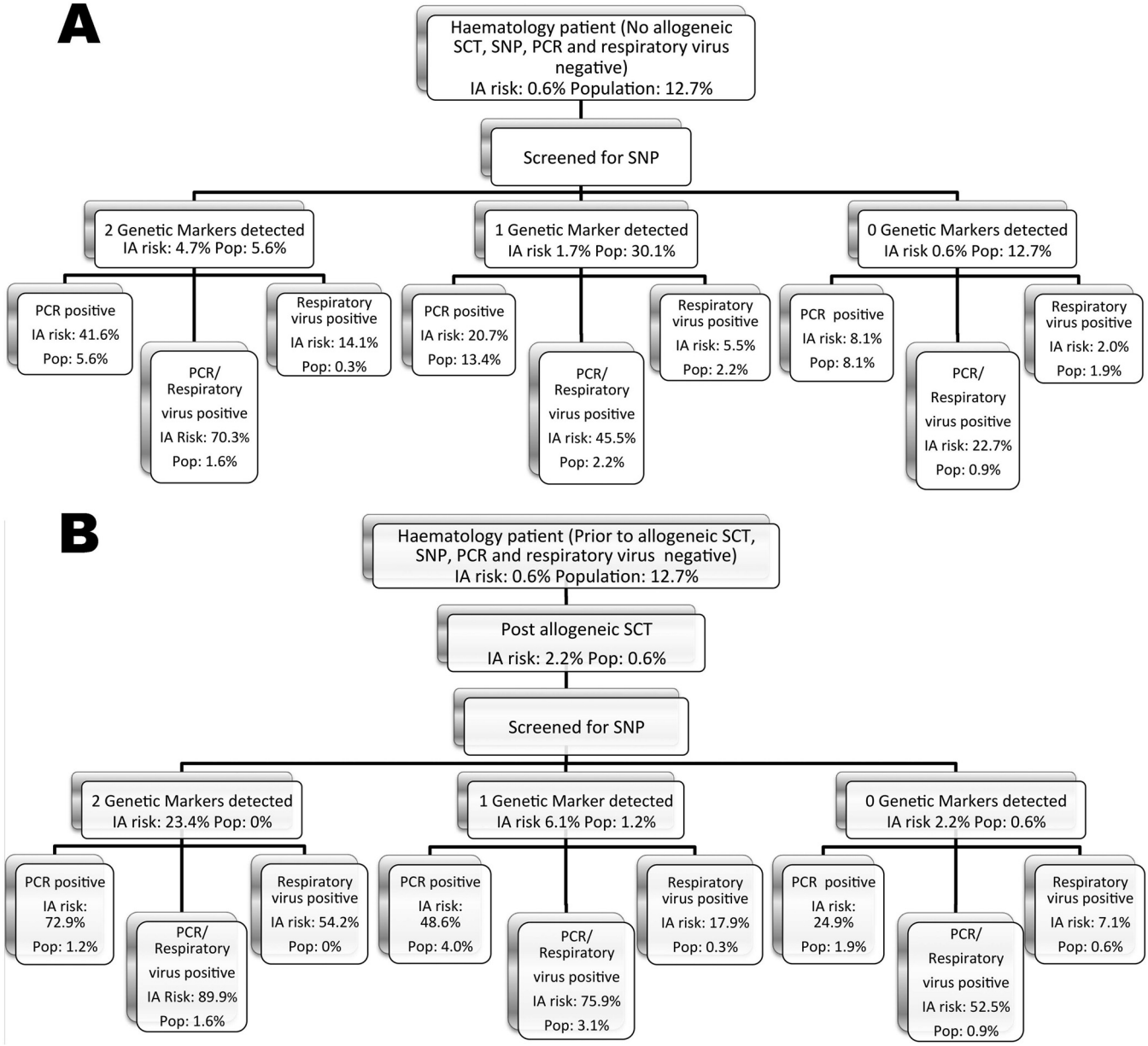

FIG 1 (A) The risk of IA associated with hematology patients not receiving, or prior to, allogeneic SCT ($n = 272$) and the influence of genetic markers, respiratory virus infection, and *Aspergillus* PCR results as determined by multilogistic regression analysis. Percent population (Pop) refers to the proportion of the total hematology patient cohort ($n = 322$). PCR positivity was defined by using a single positive PCR result as significant. (B) The risk of IA associated with hematology patients receiving allogeneic SCT ($n = 50$) and the influence of genetic markers, respiratory virus infection, and *Aspergillus* PCR results as determined by multilogistic regression analysis. Percent population (Pop) refers to the proportion of the total hematology patient cohort ($n = 322$). PCR positivity was defined by using a single positive PCR result as significant.

risk factors generated a mean probability of developing IA across the combined hematology population of 56.7%. However, the probability of developing IA varied according to the combination of the three risk factors, and in non-allogeneic SCT patients (already missing one risk factor), the presence of four risk factors (genetic markers, *Aspergillus* PCR positivity, and respiratory virus infection) was required to achieve a risk of >50% ($n = 5$ patients). In this population, the risk associated with three factors ranged from 14.1 to 45.5% (Fig. 1A). If a minimum of three risk factors was required, the mean probability of developing IA in the non-allogeneic SCT population alone was 48.9%. In the allogeneic SCT population, the probability of developing IA when a patient had three risk factors ranged from 17.9 to 52.5% (Fig. 1B). For the

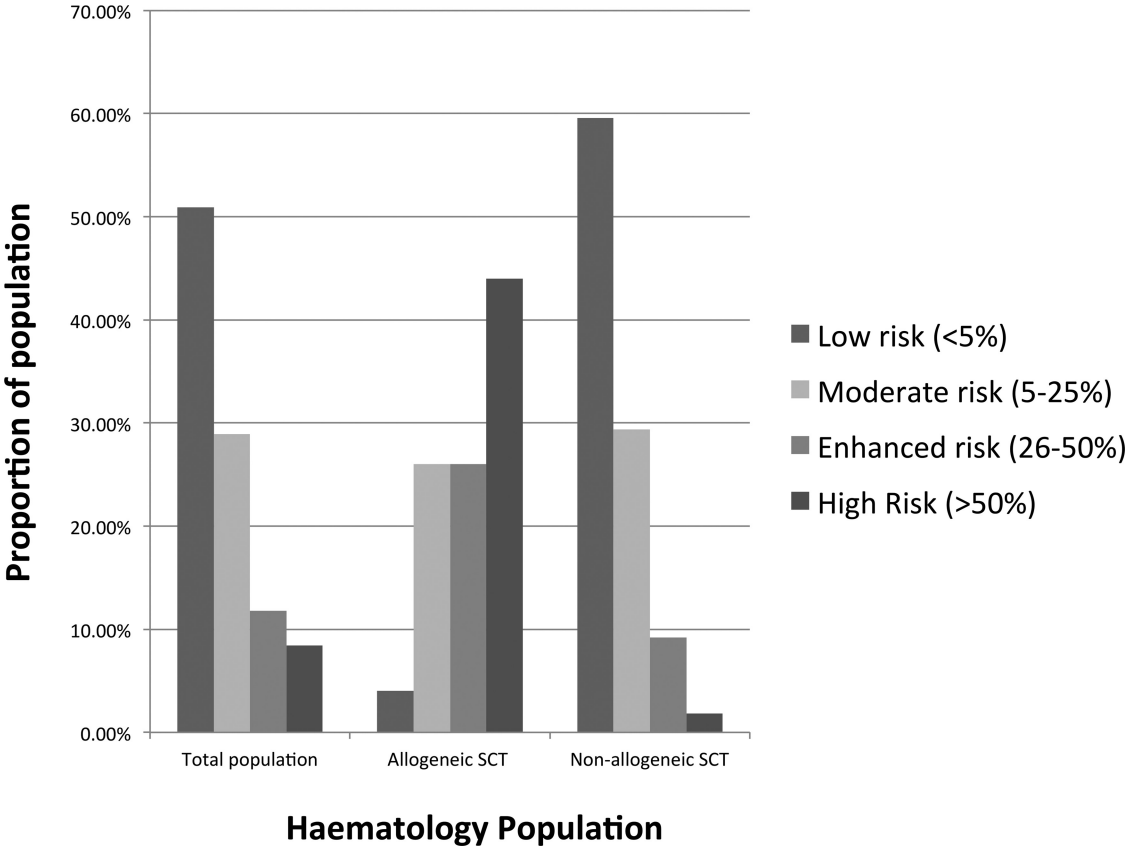

FIG 2 The distribution of risk of developing IA in a combined hematology population ($n = 322$), hematology patients after allogeneic SCT ($n = 50$), and hematology patients without allogeneic SCT ($n = 272$).

allogeneic SCT population alone, the mean probability of developing IA using a threshold of at least three positive risk factors was 63.9%. If at least four risk factors were required, the probability of developing IA in the allogeneic SCT population alone was 83.3%.

When combining the significant variables, it was decided to take a strategic approach based on the probable time line of evidence available in the clinic. SNP analysis could identify patients with genetic susceptibility to IA who could benefit from disease-

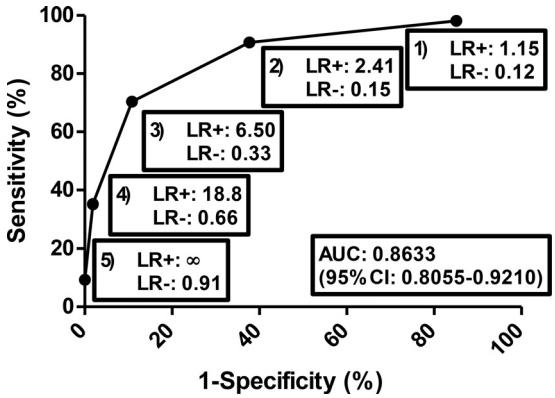

FIG 3 ROC curve for the final predictive model containing allogeneic SCT, SNPs in dectin-1 and DC-SIGN haplotype, respiratory virus infection, and *Aspergillus* PCR positivity as risk factors associated with IA. The thresholds for determining sensitivity/specificity are as follows: 1, one variable positive; 2, two variables positive; 3, three variables positive; 4, four variables positive; 5, all variables positive. AUC, area under the curve.

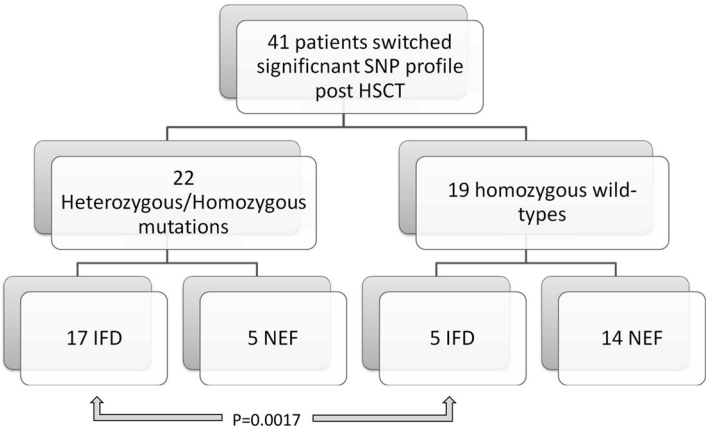

FIG 4 Switches in allele type at the SNPs significantly associated with increased risk of IA. HSCT, hematopoietic SCT.

preventive strategies (either active mold prophylaxis or PCR/GM EIA screening and preemptive treatment). Alternatively, a low probability of IA (<5%) could lead to a diagnostic approach with biomarker testing only when disease was clinically suspected.

In this study, 41 SCT patients changed alleles at loci where SNPs were significantly associated with IA (Fig. 4). Nineteen patients had SNPs removed after SCT, and 5 developed IA, whereas 22 patients had SNPs introduced after SCT and significantly more ($n = 17$; $P = 0.0017$) developed IA. Further studies are needed, but this suggests a need to screen either donors or patients after SCT to determine the risk. There is the potential to remove high-risk SNPs by finding a suitable donor and to prevent the introduction of SNPs into patients with wild-type alleles before SCT.

After SCT, the presence of high-risk SNPs increased the risk of IA >5% and biomarker screening is required (Fig. 2). If the patient is subsequently PCR positive, the risk of IA exceeds 48% and respiratory virus infection increased this further to >75.9%. These patients should be screened by PCR/GM EIA and treated preemptively to reduce the mortality rate due to delayed diagnosis of IA disease (20).

Choosing optimal genetic markers can be difficult. Many have ethnic or geographic linkage and may be unique to a local population, limiting the widespread potential of the generic strategies. The SNPs used in this study, targeting an ~95% Caucasian Welsh population ($n = 322$, 54 with IA), confirm the findings of a previous Spanish study ($n = 182$, 57 with IA) (14). Further confirmation of the applicability of these SNPs in additional ethnicities is required, and the application of novel SNPs must be investigated. Genome-wide association studies and high-throughput next-generation sequencing will identify further SNPs associated with IA and provide large amounts of specific information with minimal effort.

The DC-SIGN SNPs in both this study and the previous Spanish study were associated with IA, but it is important to exclude the possibility of confounding factors (14). DC-SIGN is reported to have roles in HIV, hepatitis C virus, and CMV infections (21–23). The present study found no correlation between CMV infection and the presence of either DC-SIGN SNP (rs114, $P = 0.9314$; rs724, $P = 0.4370$), but larger studies are needed. The heterogeneity of the patient population, but even more the limited number of certain clinical conditions, including allogeneic SCT, will limit the ability of this study to determine all risk factors. Large-scale multicenter studies targeting specific populations (e.g., transplant versus no transplant) are required to determine conclusive findings. A further limitation of this study is the limited number of proven cases ($n = 6$). However, the number of probable cases compensates for this, and while the level of confidence of diagnosis is less than that for proven IA, it highlights the difficulty in diagnosing IA and represents the usual level of diagnosis attained in the clinical setting. Furthermore, cases of probable IA are regularly accepted in clinical trials of antifungal

therapy and their inclusion is equally applicable in the present study. PCR is currently excluded from the revised EORTC/MSG definitions of IFD (16). Data from the audit of this cohort showed most possible IA cases (GM negative be definition) to be *Aspergillus* PCR positive (2). The likely inclusion of PCR in the second revision of the definitions will therefore increase the number of probable cases in this study but also provide an alternative mycological criterion allowing us to incorporate GM testing into the model without the concern of incorporation bias.

In conclusion, this proof-of-concept study shows that genetic markers, in combination with clinical risk and early biomarker positivity, can facilitate the stratification of patients according to their IA risk. If the probability of developing IA passes 50%, then the use of preemptive therapy is justified, and in this study, only 8.3% (27/322) of the population, mainly allogeneic SCT patients ($n = 22$), would receive therapy (Fig. 2). It highlights what can be achieved to improve the management of patients at risk of difficult-to-manage fungal diseases and is an attempt to translate preclinical research into a clinical setting (24). For this type of approach to be of use, it must be easily accessible, dynamic to change, and simple to use with respect to data input and processing. These systems can be applied to portable electronic devices to permit real-time clinical decision making, individual patient management, and a strategic evidence-based approach to the management of IA or other difficult-to-diagnose diseases. Further studies are needed to determine the range of genetic markers associated with IA, and while a recent large-scale study by Fisher et al. investigated the relevance of previously documented SNPs by using microarray analysis, the application of whole-genome sequencing will be essential for this purpose (25).

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REFERENCES

1. Donnelly JP, Maertens J. 2013. The end of the road for empirical antifungal treatment? Lancet Infect Dis 13:470–472. https://doi.org/10.1016/S1473-3099(13)70112-9.
2. Barnes RA, Stocking K, Bowden S, Poynton MH, White PL. 2013. Prevention and diagnosis of invasive fungal disease in high-risk patients within an integrative care pathway. J Infect 67:206–214. https://doi.org/10.1016/j.jinf.2013.04.020.
3. Morrissey CO, Chen SC, Sorrell TC, Milliken S, Bardy PG, Bradstock KF, Szer J, Halliday CL, Gilroy NM, Moore J, Schwarer AP, Guy S, Bajel A, Tramontana AR, Spelman T, Slavin MA, Australasian Leukaemia Lymphoma Group, Australia and New Zealand Mycology Interest Group. 2013. Galactomannan and PCR versus culture and histology for directing use of antifungal treatment for invasive aspergillosis in high-risk hematology patients: a randomised controlled trial. Lancet Infect Dis 13:519–528. https://doi.org/10.1016/S1473-3099(13)70076-8.
4. Aguado JM, Vázquez L, Fernández-Ruiz M, Villaescusa T, Ruiz-Camps I, Barba P, Silva JT, Batlle M, Solano C, Gallardo D, Heras I, Polo M, Varela R, Vallejo C, Olave T, López-Jiménez J, Rovira M, Parody R, Cuenca-Estrella M, PCRAGA Study Group, Spanish Stem Cell Transplantation Group, Study Group of Medical Mycology of the Spanish Society of Clinical Microbiology and Infectious Diseases, Spanish Network for Research in Infectious Diseases. 2015. Serum galactomannan versus a combination of galactomannan and PCR-based *Aspergillus* DNA detection for early therapy of invasive aspergillosis in high-risk hematological patients: a randomized controlled trial. Clin Infect Dis 60:405–414. https://doi.org/10.1093/cid/ciu833.
5. Schwarzinger M, Sagaon-Teyssier L, Cabaret O, Bretagne S, Cordonnier C, Investigators PREVERT. 2013. Performance of serum biomarkers for the early detection of invasive aspergillosis in febrile, neutropenic patients: a multi-state model. PLoS One 8:e65776. https://doi.org/10.1371/journal.pone.0065776.
6. Barnes PD, Marr KA. 2007. Risks, diagnosis and outcomes of invasive fungal infections in haematopoietic stem cell transplant recipients. Br J Haematol 139:519–531. https://doi.org/10.1111/j.1365-2141.2007.06812.x.
7. Baddley JW. 2011. Clinical risk factors for invasive aspergillosis. Med Mycol 49(Suppl 1):S7–S12. https://doi.org/10.3109/13693786.2010.505204.
8. Castagnola E, Viscoli C. 2009. Invasive aspergillosis in malignancy and stem cell transplant recipients, p 519–530. *In* Latge J-P, Steinbach WJ (ed), *Aspergillus fumigatus* and aspergillosis. ASM Press, Washington, DC.
9. Hardison SE, Brown GD. 2012. C-type lectin receptors orchestrate antifungal immunity. Nat Immunol 13:817–822. https://doi.org/10.1038/ni.2369.
10. Caira M, Mancinelli M, Leone G, Pagano L. 2011. Invasive aspergillosis in acute leukemias: old and new risk factors and new epidemiological trends.

Med Mycol 49(Suppl 1):S13–S16. https://doi.org/10.3109/13693786.2010.509138.
11. Mennink-Kersten MA, Ruegebrink D, Wasei N, Melchers WJ, Verweij PE. 2006. In vitro release by *Aspergillus fumigatus* of galactofuranose antigens, 1,3-beta-D-glucan, and DNA, surrogate markers used for diagnosis of invasive aspergillosis. J Clin Microbiol 44:1711–1718. https://doi.org/10.1128/JCM.44.5.1711-1718.2006.
12. Springer J, Morton CO, Perry M, Heinz WJ, Paholcsek M, Alzheimer M, Rogers TR, Barnes RA, Einsele H, Loeffler J, White PL. 2013. Multicenter comparison of serum and whole-blood specimens for detection of *Aspergillus* DNA in high-risk hematological patients. J Clin Microbiol 51:1445–1450. https://doi.org/10.1128/JCM.03322-12.
13. White PL, Wiederhold NP, Loeffler J, Najvar LK, Melchers W, Herrera M, Bretagne S, Wickes B, Kirkpatrick WR, Barnes RA, Donnelly JP, Patterson TF. 2016. Comparison of nonculture blood-based tests for diagnosing invasive aspergillosis in an animal model. J Clin Microbiol 54:960–966. https://doi.org/10.1128/JCM.03233-15.
14. Sainz J, Lupianez CB, Segura-Catena J, Vazquez L, Ríos R, Oyonarte S, Hemminki K, Försti A, Jurado M. 2012. Dectin-1 and DC-SIGN polymorphisms associated with invasive pulmonary aspergillosis infection. PLoS One 7:e32273. https://doi.org/10.1371/journal.pone.0032273.
15. Barnes RA, White PL, Bygrave C, Evans N, Healy B, Kell J. 2009. Clinical impact of enhanced diagnosis of invasive fungal disease in high-risk haematology and stem cell transplant patients. J Clin Pathol 62:64–69. https://doi.org/10.1136/jcp.2008.058354.
16. De Pauw B, Walsh TJ, Donnelly JP, Stevens DA, Edwards JE, Calandra T, Pappas PG, Maertens J, Lortholary O, Kauffman CA, Denning DW, Patterson TF, Maschmeyer G, Bille J, Dismukes WE, Herbrecht R, Hope WW, Kibbler CC, Kullberg BJ, Marr KA, Muñoz P, Odds FC, Perfect JR, Restrepo A, Ruhnke M, Segal BH, Sobel JD, Sorrell TC, Viscoli C, Wingard JR, Zaoutis T, Bennett JE, European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group, National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG) Consensus Group. 2008. Revised definitions of invasive fungal disease from the European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG) Consensus Group. Clin Infect Dis 46:1813–1821. https://doi.org/10.1086/588660.
17. White PL, Bretagne S, Klingspor L, Melchers WJ, McCulloch E, Schulz B, Finnstrom N, Mengoli C, Barnes RA, Donnelly JP, Loeffler J, European *Aspergillus* PCR Initiative. 2010. *Aspergillus* PCR: one step closer to standardization. J Clin Microbiol 48:1231–1240. https://doi.org/10.1128/JCM.01767-09.
18. White PL, Linton CJ, Perry MD, Johnson EM, Barnes RA. 2006. The evolution and evaluation of a whole blood polymerase chain reaction assay for the detection of invasive aspergillosis in hematology patients in a routine clinical setting. Clin Infect Dis 42:479–486. https://doi.org/10.1086/499949.
19. Chai LY, de Boer MG, van der Velden WJ, Plantinga TS, van Spriel AB, Jacobs C, Halkes CJ, Vonk AG, Blijlevens NM, van Dissel JT, Donnelly PJ, Kullberg BJ, Maertens J, Netea MG. 2011. The Y238X stop codon polymorphism in the human beta-glucan receptor dectin-1 and susceptibility to invasive aspergillosis. J Infect Dis 203:736–743. https://doi.org/10.1093/infdis/jiq102.
20. von Eiff M, Roos N, Schulten R, Hesse M, Zühlsdorf M, van de Loo J. 1995. Pulmonary aspergillosis: early diagnosis improves survival. Respiration 62:341–347. https://doi.org/10.1159/000196477.
21. Plazolles N, Humbert J-M, Vachot L, Verrier B, Hocke C, Halary F. 2011. Pivotal advance: the promotion of soluble DC-SIGN release by inflammatory signals and its enhancement of cytomegalovirus-mediated *cis*-infection of myeloid dendritic cells. J Leukoc Biol 89:329–342. https://doi.org/10.1189/jlb.0710386.
22. Lozach PY, Amara A, Bartosch B, Virelizier JL, Arenzana-Seisdedos F, Cosset FL, Altmeyer R. 2004. C-type lectins L-SIGN and DC-SIGN capture and transmit infectious hepatitis C virus pseudotype particles. J Biol Chem 279:32035–32045. https://doi.org/10.1074/jbc.M402296200.
23. Geijtenbeek TB, Kwon DS, Torensma R, van Vliet SJ, van Duijnhoven GC, Middel J, Cornelissen IL, Nottet HS, KewalRamani VN, Littman DR, Figdor CG, van Kooyk Y. 2000. DC-SIGN, a dendritic cell-specific HIV-1-binding protein that enhances trans-infection of T cells. Cell 100:587–597. https://doi.org/10.1016/S0092-8674(00)80694-7.
24. Head MG, Fitchett JR, Atun R, May RC. 2014. Systematic analysis of funding awarded for mycology research to institutions in the UK, 1997–2010. BMJ Open 4:e004129. https://doi.org/10.1136/bmjopen-2013-004129.
25. Fisher CE, Hohl TM, Fan W, Storer BE, Levine DM, Zhao LP, Martin PJ, Warren EH, Boeckh M, Hansen JA. 2017. Validation of single nucleotide polymorphisms in invasive aspergillosis following hematopoietic cell transplantation. Blood 129:2693–2701. https://doi.org/10.1182/blood-2016-10-743294.