ORIGINAL ARTICLE

Genetic PTX3 Deficiency and Aspergillosis in Stem-Cell Transplantation

Cristina Cunha, Ph.D., Franco Aversa, M.D., João F. Lacerda, M.D., Ph.D., Alessandro Busca, M.D., Oliver Kurzai, M.D., Matthias Grube, M.D., Jürgen Löffler, Ph.D., Johan A. Maertens, M.D., Ph.D., Alain S. Bell, Ph.D., Antonio Inforzato, Ph.D., Elisa Barbati, Ph.D., Bruno Almeida, Ph.D., Pedro Santos e Sousa, M.D., Anna Barbui, M.D., Leonardo Potenza, M.D., Ph.D., Morena Caira, M.D., Ph.D., Fernando Rodrigues, Ph.D., Giovanni Salvatori, Ph.D., Livio Pagano, M.D., Mario Luppi, M.D., Ph.D., Alberto Mantovani, M.D., Andrea Velardi, M.D., Luigina Romani, M.D., Ph.D., and Agostinho Carvalho, Ph.D.

ABSTRACT

BACKGROUND

The soluble pattern-recognition receptor known as long pentraxin 3 (PTX3) has a nonredundant role in antifungal immunity. The contribution of single-nucleotide polymorphisms (SNPs) in *PTX3* to the development of invasive aspergillosis is unknown.

METHODS

We screened an initial cohort of 268 patients undergoing hematopoietic stem-cell transplantation (HSCT) and their donors for *PTX3* SNPs modifying the risk of invasive aspergillosis. The analysis was also performed in a multicenter study involving 107 patients with invasive aspergillosis and 223 matched controls. The functional consequences of *PTX3* SNPs were investigated in vitro and in lung specimens from transplant recipients.

RESULTS

Receipt of a transplant from a donor with a homozygous haplotype (h2/h2) in *PTX3* was associated with an increased risk of infection, in both the discovery study (cumulative incidence, 37% vs. 15%; adjusted hazard ratio, 3.08; P=0.003) and the confirmation study (adjusted odds ratio, 2.78; P=0.03), as well as with defective expression of PTX3. Functionally, PTX3 deficiency in h2/h2 neutrophils, presumably due to messenger RNA instability, led to impaired phagocytosis and clearance of the fungus.

CONCLUSIONS

Genetic deficiency of PTX3 affects the antifungal capacity of neutrophils and may contribute to the risk of invasive aspergillosis in patients treated with HSCT. (Funded by the European Society of Clinical Microbiology and Infectious Diseases and others.)

The authors' affiliations are listed in the Appendix. Address reprint requests to Dr. Carvalho at the Department of Experimental Medicine and Biochemical Sciences, University of Perugia, Polo Unico Sant'Andrea delle Fratte, 06132 Perugia, Italy, or at agostinho.carvalho@unipg.it.

N Engl J Med 2014;370:421-32.
DOI: 10.1056/NEJMoa1211161

Long pentraxin 3 (PTX3) is a soluble pattern-recognition receptor produced by phagocytes and nonimmune cells at sites of inflammation or injury. In addition to its major role in female fertility and vascular biology,[1] PTX3 has a nonredundant role in modulating various effector pathways involved in immune resistance to *Aspergillus fumigatus*, including activating innate immune cells[2] and driving protective adaptive immunity.[3] PTX3 forms complexes on the conidial surface of the fungus and acts as an opsonin, enhancing recognition and phagocytosis of conidia through mechanisms that depend on Fcγ receptor, CD11b, and complement.[4] The interaction of PTX3 with the yeast phase of *Candida albicans*[5] and *Paracoccidioides brasiliensis*[6] has also been reported.

Mononuclear phagocytes and dendritic cells synthesize PTX3 de novo in response to microbial moieties or inflammatory signals. In contrast, PTX3 is stored in a ready-made form in granules from mature polymorphonuclear leukocytes, and its secretion and localization to extracellular traps promote the control of *A. fumigatus* infection in vivo.[7] Therefore, it is not surprising that Ptx3 deficiency renders mice susceptible to pulmonary aspergillosis because of the defective recognition of conidia by neutrophils, alveolar macrophages, and dendritic cells.[3,4] The susceptibility phenotype is associated with impaired activation of a protective type 1 helper T-cell (Th1) antifungal response coupled with a detrimental type 2 helper T-cell response.[3] The administration of exogenous Ptx3 in mice that have undergone bone marrow transplantation reverts the susceptibility to disease by restoring the Th1 response[3]; the administration of exogenous Ptx3 also restrains the type 17 helper T-cell response and associated pathogenic inflammation induced during infection in experimental models of chronic granulomatous disease.[8] The protective effect of exogenous Ptx3 is further substantiated by the capacity of the protein to improve the efficacy of antifungal therapy in mice when the two treatments are administered in combination.[9,10]

The pivotal role of PTX3 in innate antifungal immunity[11] makes the protein an attractive candidate for studies of genetic susceptibility to fungal diseases in high-risk patients, such as those undergoing hematopoietic stem-cell transplantation (HSCT). Invasive aspergillosis may develop as a complication in 5 to 15% of patients undergoing allogeneic HSCT.[12-14] We screened patients undergoing HSCT and their donors for single-nucleotide polymorphisms (SNPs) in the *PTX3* gene and assessed the association of identified SNPs with susceptibility to invasive aspergillosis and impaired innate antifungal immunity.

METHODS

PATIENTS AND STUDY PROCEDURES

All adult patients with hematologic disorders who were undergoing allogeneic HSCT at the Hospital of the University of Perugia, in Perugia, Italy, between 2003 and 2011, and their respective donors (who were related in 98% of cases), were eligible for the discovery study. Among 430 transplantations performed in this period, 346 recipient–donor pairs with available DNA and patient-level data were identified. Exclusion criteria were participation in clinical studies (64 patients),[15] infection with invasive molds other than aspergillus species (12), and pretransplantation infection with molds (2). Fifty-one cases of invasive aspergillosis were classified as "probable" (in 26 patients) or "proven" (in 25 patients), according to the revised standard criteria from the European Organization for Research and Treatment of Cancer–Mycoses Study Group[16] (Table 1). Among these cases, 9 (18%) occurred in the neutropenic period and 42 (82%) after engraftment. Transplantation procedures were performed as previously described.[17] Graft-versus-host disease (GVHD) and cytomegalovirus disease were diagnosed according to standard criteria.[18,19] Conditioning regimens and prophylaxis are described in the Supplementary Appendix, available with the full text of this article at NEJM.org. The study was approved by the Umbria Regional Hospital Ethics Committee in Perugia, and patients and donors provided informed written consent for data collection, DNA and cell storage, and their use for diagnostic and research purposes.

The methods used for the confirmation study (Table S1 in the Supplementary Appendix) and the genetic and functional analyses are detailed in the Supplementary Appendix.

STATISTICAL ANALYSIS

In the analysis of the discovery data set, the probability of invasive aspergillosis according to *PTX3* variants was determined with the use of the

Table 1. Characteristics of Transplant Recipients in the Discovery Study.*

Clinical Variable	Invasive Aspergillosis (N=51)	No Invasive Aspergillosis (N=217)	P Value†
Age at transplantation — no. (%)			
<40 yr	26 (51)	112 (52)	
≥40 yr	25 (49)	105 (48)	1.00
Sex — no. (%)			
Male	23 (45)	107 (49)	
Female	28 (55)	110 (51)	0.64
Sex of donor–recipient pair — no. (%)			
Female–male	10 (20)	58 (27)	
Other	41 (80)	159 (73)	0.37
Transplantation type — no. (%)			
Transplant from HLA-matched related donor	14 (27)	90 (42)	
Transplant from HLA-mismatched related donor‡	37 (73)	122 (56)	0.06
Transplant from HLA-matched unrelated donor	0	5 (2)	0.62
Transplant from HLA-mismatched unrelated donor	0	0	1.00
Underlying disease — no. (%)			
Acute leukemia	31 (61)	152 (70)	
Lymphoma or myeloma	14 (27)	53 (24)	0.58
Chronic leukemia	4 (8)	11 (5)	0.48
Other	2 (4)	1 (1)	0.08
Disease stage — no. (%)			
First complete remission	14 (27)	73 (34)	
Second or subsequent remission, or relapse	37 (73)	144 (66)	0.41
Myeloablative conditioning regimen — no. (%)			
Total-body irradiation	43 (84)	150 (69)	
No total-body irradiation	8 (16)	67 (31)	0.04
CMV serostatus of donor and recipient — no. (%)			
Donor and recipient CMV-negative or donor CMV-positive and recipient CMV-negative	5 (10)	27 (12)	
Donor CMV-negative and recipient CMV-positive or donor and recipient CMV-positive	46 (90)	190 (88)	0.65
Duration of neutropenia — median no. of days (range)§	16 (10–24)	16 (10–40)	0.39
Acute GVHD, grade II, III, or IV — no. (%)	5 (10)	10 (5)	0.17
Antifungal prophylaxis — no. (%)¶			
Liposomal amphotericin B	49 (96)	177 (82)	
Fluconazole	2 (4)	40 (18)	0.02

* CMV denotes cytomegalovirus, and GVHD graft-versus-host disease.
† P values were calculated by Fisher's exact probability t-test, or by Student's t-test for continuous variables.
‡ Recipients of transplants from mismatched related donors received rabbit antithymocyte globulin as part of their pretransplantation conditioning regimen.
§ Neutropenia was defined as 0.1×10^9 neutrophils per liter or less.
¶ Antifungal prophylaxis consisted of fluconazole for standard-risk patients and liposomal amphotericin B for high-risk patients. At the time of allogeneic hematopoietic stem-cell transplantation, patients were considered to be at high risk for invasive fungal disease if they were receiving a transplant from an HLA-mismatched related donor or if they were receiving a transplant from an HLA-identical sibling and had undergone conditioning that included total-body irradiation. Patients were considered to be at standard risk if they were receiving a transplant from an HLA-identical sibling and had not undergone conditioning with total-body irradiation.

cumulative incidence method, and *PTX3* status among patients with and those without infection was compared with the use of Gray's test.[20] Cumulative incidences were computed with R software, version 2.10.1 (cmprsk package),[21] with censoring of data at the date of the last follow-up visit, and with relapse and death as competing risks. A period of 24 months was chosen to include all cases in the discovery cohort. Overall survival, defined as the time from transplantation to death from any cause, was estimated with the use of the Kaplan–Meier method and assessed according to *PTX3* status with the use of the log-rank test. Additional details on statistical methods are provided in the Supplementary Appendix.

RESULTS

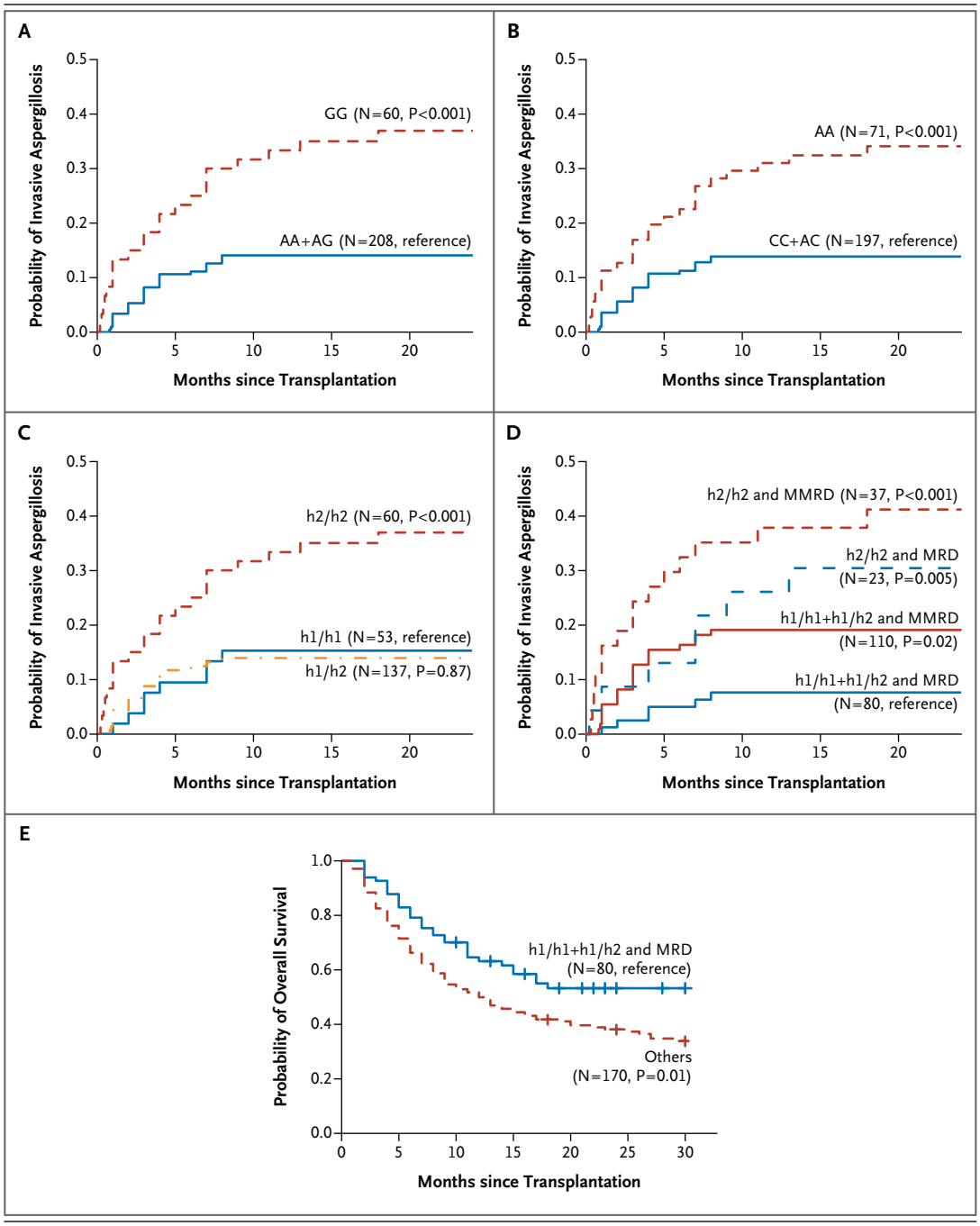
GENETIC VARIANTS IN DONOR *PTX3* AND THE RISK OF INVASIVE ASPERGILLOSIS

The *PTX3* gene was analyzed for 22 SNPs spanning approximately 25 kb on chromosome 3 (Table S2 in the Supplementary Appendix). We assessed the risk of invasive aspergillosis according to recipient or donor *PTX3* genotypes by estimating the cumulative incidence of infection among transplant recipients 24 months after transplantation. Several SNPs in the donors were associated with a risk of invasive aspergillosis in the recipients. We did not observe an association between SNPs in recipients and a risk of infection (Table S3 in the Supplementary Appendix). Among these SNPs, only +281A/G, +734A/C (D48A), and +1449A/G remained significantly associated with invasive aspergillosis after correction for multiple testing. The cumulative incidence of invasive aspergillosis among patients with +281A/G and +1449A/G was 37% for genotype GG and 14% for genotypes AA and AG combined (P<0.001; adjusted P=0.002) (Fig. 1A); the cumulative incidence of invasive aspergillosis among patients with +734A/C was 34% for genotype AA and 14% for genotypes AC and CC combined (P<0.001; adjusted P=0.005) (Fig. 1B).

Analysis of linkage disequilibrium between markers showed the existence of three distinct linkage-disequilibrium blocks (Fig. S1 in the Supplementary Appendix). Haplotype analysis revealed that an absence of donor haplotypes T-A (block 1), A-A (block 2), and A-C (block 3) was associated with an increased risk of infection among recipients after transplantation (Table S4 in the Supplementary Appendix), even though the cumulative incidence of infection among patients with A-C/A-C (block 1) donors was not significantly different from the incidence among patients with T-A/T-A donors and the cumulative incidence of infection among patients with T-G/T-G (block 2) donors was not significantly different from the incidence among patients with A-A/A-A donors (Fig. S2 in the Supplementary Appendix). However, the cumulative incidence of infection with transplants from G-A/G-A (hereafter referred to as h2/h2) block 3 donors was significantly higher than that with transplants from A-C/A-C (h1/h1) donors and A-C/G-A (h1/h2) donors (37%

Figure 1 (facing page). Cumulative Incidence of Invasive Aspergillosis and Estimated Overall Survival after Hematopoietic Stem-Cell Transplantation in 268 Patients in the Discovery Cohort, According to Donor *PTX3* Variants.

Panels A, B, C, and D show the probability of proven or probable invasive aspergillosis, with censoring of data at 24 months and with relapse and death as competing events. P values were calculated with the use of Gray's test. Panel E shows the probability of overall survival, with censoring of data at 30 months. Kaplan–Meier estimates were used to calculate survival curves, and P values were determined with the use of the log-rank test. Panel A shows the cumulative incidence of invasive aspergillosis in transplant recipients according to donor +281A/G genotypes. The same plot also shows donor +1449A/G, since it displayed complete linkage disequilibrium (r^2=1) with +281A/G among cases of invasive aspergillosis. Panel B shows the cumulative incidence of invasive aspergillosis according to donor +734A/C (D48A) genotypes. Panel C shows the cumulative incidence of invasive aspergillosis according to donor haplotypes h1/h1 (A-C/A-C), h1/h2 (A-C/G-A), and h2/h2 (G-A/G-A). The +281A/G and +1449A/G SNPs are in strong linkage disequilibrium and can be used interchangeably within haplotype block 3. Panel D shows the cumulative incidence of invasive aspergillosis according to donor haplotypes (h1/h1, as well as h1/h2 and h2/h2) and type of donor (HLA-matched related donor [MRD] or HLA-mismatched related donor [MMRD]). Haplotypes h1/h1 and h1/h2 were combined in a single category, since they conferred a similar risk of invasive aspergillosis, as shown in Panel C. P=0.75 for the test for interaction between the *PTX3* haplotypes and the type of transplant. Panel E shows overall survival according to donor *PTX3* variants. Shown are the results for patients who received transplants from MRDs with the h1/h1 or h1/h2 haplotype versus patients who received transplants from other donors (MMRDs with the h1/h1 or h1/h2 haplotype and either MRDs or MMRDs with the h2/h2 haplotype).



vs. 15% and 37% vs. 14%, respectively; P<0.001; adjusted P=0.001); the cumulative incidence of infection with transplants from A-C/G-A (h1/h2) donors was similar to that with transplants from A-C/A-C (h1/h1) donors (P=0.87) (Fig. 1C).

With regard to rare haplotypes, an 18% incidence of invasive aspergillosis was observed among recipients of transplants from A-A/A-A donors (P=0.85), whereas no cases were observed among recipients of transplants from donors with the A-C/A-A haplotype (P=0.31) (Fig. S3 in the Supplementary Appendix).

In the final multivariate SNP models, the adjusted hazard ratio for invasive aspergillosis

with either donor genotype +281GG or donor genotype +1449GG was 2.92 (95% confidence interval [CI], 1.69 to 5.05; P<0.001), and the hazard ratio with donor genotype +734AA was 2.62 (95% CI, 1.52 to 4.54; P<0.001) (Table 2). In the final haplotype model, the hazard ratio for invasive aspergillosis with h2/h2 carriage was 3.08 (95% CI, 1.47 to 6.44; P=0.003). Risk estimates were similar when patients with "possible" (as opposed to "probable" or "proven") aspergillosis were excluded and when patients with invasive fungal disease other than invasive aspergillosis were included (Fig. S4 in the Supplementary Appendix).

The characteristics of the patients in the confirmation case–control study were similar to those of the patients in the initial study (Table S1 in the Supplementary Appendix), but the confirmation study included recipients of transplants from related donors and recipients of transplants from unrelated donors. Haplotype frequencies in the confirmation population were similar to those in the discovery population (Table S5 in the Supplementary Appendix). The significant association between donor haplotype h2/h2 and the risk of invasive aspergillosis in the discovery study (T-cell–depleted grafts and absence of GVHD and its prophylaxis) was also observed in the confirmation study (T-cell–repleted grafts and presence of GVHD and use of prophylaxis) (adjusted odds ratio, 2.78; 95% CI, 1.22 to 8.93; P=0.03) (Table 2) but not in a second, independent confirmation study involving a cohort of patients with prolonged neutropenia who had not undergone HSCT (Table S6 in the Supplementary Appendix).

RISK OF INVASIVE ASPERGILLOSIS ACCORDING TO DONOR HAPLOTYPE AND HLA-MATCHING STATUS

Given that donor *PTX3* haplotypes and HLA profile are pretransplantation predictors of the risk of invasive aspergillosis, we stratified patients in the discovery cohort according to the type of donor (HLA-matched related donor vs. HLA-mismatched related donor) and haplotype (h2/h2 vs. h1/h1 or h1/h2). Among transplant recipients whose donors had the h1/h1 or h1/h2 haplotype, the cumulative incidence of invasive aspergillosis increased from 8% among recipients with matched related donors to 19% among those with mismatched related donors (P=0.02) (Fig. 1D). However, in the presence of the h2/h2 haplotype, the risk increased further, from 30% among recipients with matched related donors (P=0.005 for the comparison with h1/h1 or h1/h2 matched related donors) to 41% among those with mismatched related donors (P<0.001 for the comparison with h1/h1 or h1/h2 matched related donors). In a multivariate analysis, the hazard ratio for invasive aspergillosis among transplant re-

Table 2. Multivariate Analysis of the Association of Donor *PTX3* Variants with the Risk of Invasive Aspergillosis among Transplant Recipients in the Discovery and Confirmation Studies.*

Donor *PTX3* Variant	Discovery Study (N=268)		Confirmation Study (N=330)	
	Adjusted Hazard Ratio (95% CI)†	P Value	Adjusted Odds Ratio (95% CI)‡	P Value
+281A/G SNP, GG genotype	2.92 (1.69–5.05)	<0.001	2.14 (1.20–3.80)	0.01
+734A/C SNP, AA genotype	2.62 (1.52–4.54)	<0.001	1.92 (0.91–3.04)	0.07
Haplotype h2/h2	3.08 (1.47–6.44)	0.003	2.78 (1.22–8.93)	0.03

* CI denotes confidence interval, and SNP single-nucleotide polymorphism. Multivariate analyses were based on the subdistribution regression model of Fine and Gray[22] in the discovery study and on conditional logistic regression in the confirmation study. In the SNP model, the genetic variants were computed one at a time with the clinical covariates. The results for donor +281GG genotype are identical to those obtained for the +1449GG genotype because of their complete linkage disequilibrium among cases of invasive aspergillosis. The combination of genotypes AA and AG was the reference category for +281A/G, and the combination of genotypes CC and CA was the reference category for +734A/C. For the haplotype model, the reference category was h1/h1. Hazard ratios and odds ratios are for the presence versus the absence of the genotype or haplotype that confers a risk of invasive aspergillosis.

† Hazard ratios have been adjusted for HLA-matching status, use or nonuse of total-body irradiation in the myeloablative conditioning, and antifungal prophylaxis (fluconazole or liposomal amphotericin B). The only clinical variable that remained significantly associated with invasive aspergillosis in the SNP and haplotype models was receipt of a transplant from an HLA-mismatched relative (+281A/G: hazard ratio, 1.94; 95% CI, 1.07 to 3.52; P=0.03; +734A/C: hazard ratio, 1.88; 95% CI, 1.04 to 3.42; P=0.04; haplotype h2/h2: hazard ratio, 1.95; 95% CI, 1.06 to 3.58; P=0.03).

‡ Odds ratios have been adjusted for HLA-matching status and use or nonuse of total-body irradiation in the myeloablative conditioning regimen.

cipients with h1/h1 or h1/h2 mismatched related donors versus those with matched related donors was 2.46 (95% CI, 1.10 to 5.50; P=0.03). Among transplant recipients with h2/h2 donors, the hazard ratio, with h1/h1 or h1/h2 matched related donors as the reference group, was further increased, from 3.96 (95% CI, 1.49 to 10.50; P=0.006) among recipients with matched related donors to 6.02 (95% CI, 2.58 to 14.00; P<0.001) among those with mismatched related donors. Accordingly, the probability of survival at 30 months after transplantation decreased from 63% among patients who received transplants from matched related donors with haplotype h1/h1 or h1/h2 to 37% among patients who received transplants from either h1/h1 or h1/h2 mismatched related donors or from h2/h2 matched or mismatched related donors (P=0.01) (Fig. 1E).

EFFECT OF THE +734A/C SNP ON *PTX3* MESSENGER RNA FOLDING

To determine the molecular consequences of the SNPs in *PTX3* haplotype block 3, we examined the pathogenic potential of each SNP in terms of protein function. We first compared the amino acid sequence of annotated PTX3 proteins among several mammals to determine whether the +734A/C (D48A) mutation was occurring in a phylogenetically conserved site. Alignment of PTX3 sequences confirmed that the D48A substitution leading to an alanine residue in humans lies in an evolutionarily conserved region within the mammalian lineage, since all other species carried an aspartic acid residue instead (Fig. S5 in the Supplementary Appendix). Even though this mutation affected a conserved site, it was nonetheless predicted in silico as benign with respect to protein folding and structural stability. More important, when we looked at models of the effect of the +734A/C SNP on *PTX3* messenger RNA (mRNA) folding, we found it to be severely affected by the nucleotide substitution (Fig. S6 in the Supplementary Appendix), indicating altered mRNA stability. No differences in splicing were predicted for intronic SNPs (Table S7 in the Supplementary Appendix).

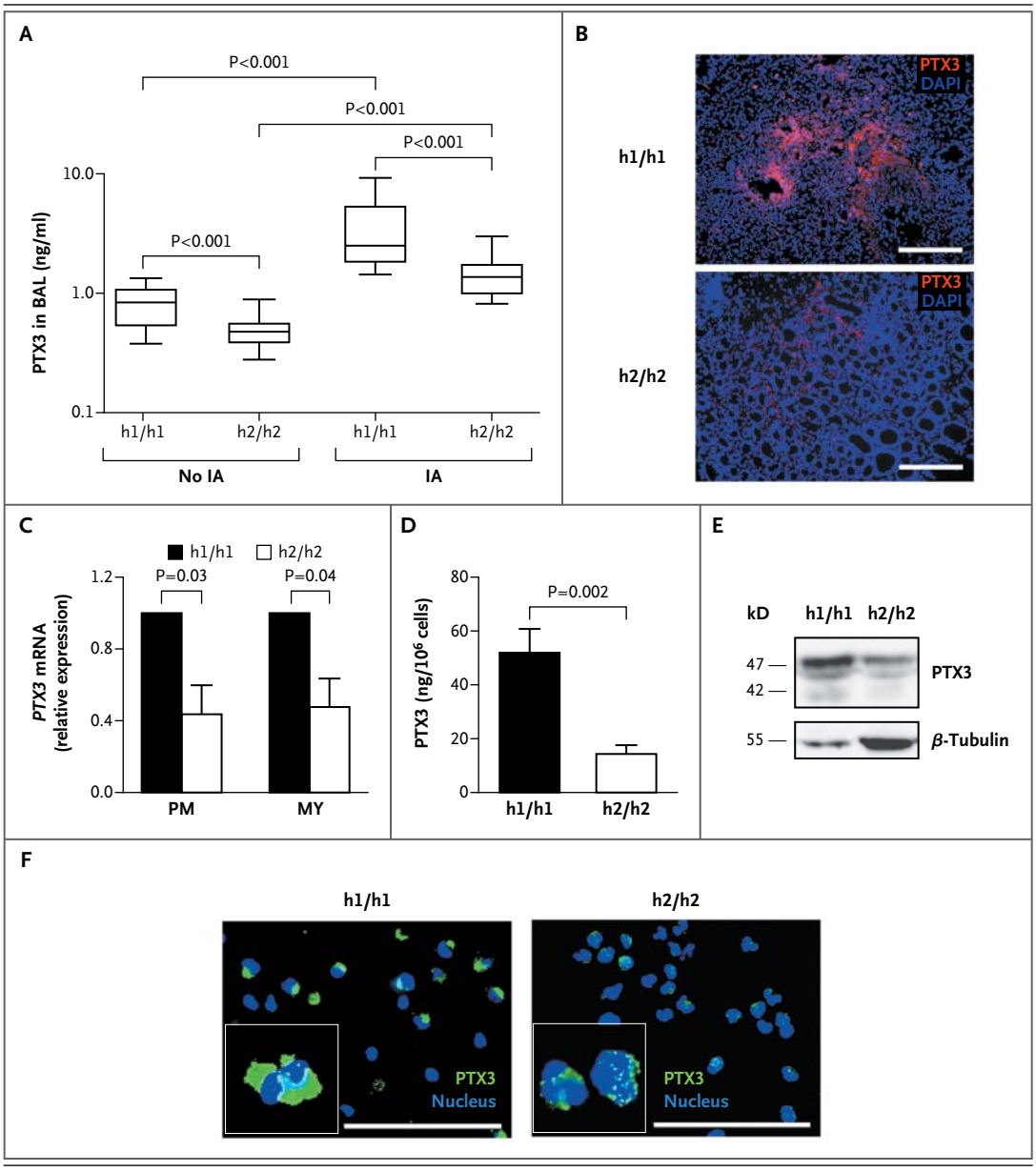
DECREASED EXPRESSION OF PTX3 WITH THE H2/H2 HAPLOTYPE

To assess the functional consequences of h2/h2, we compared PTX3 expression in vivo in the lungs of transplant recipients with invasive aspergillosis and in those without invasive aspergillosis, as well as in vitro on purified cells. We found higher levels of PTX3 in bronchoalveolar-lavage fluid, but not in plasma (Fig. S7 in the Supplementary Appendix), from patients with invasive aspergillosis than in patients without invasive aspergillosis (Fig. 2A). More important, median (±SD) PTX3 levels in bronchoalveolar-lavage fluid differed significantly according to donor haplotype both for patients with invasive aspergillosis (2.50±0.73 ng per milliliter for h1/h1 [14 patients] vs. 1.36±0.14 ng per milliliter for h2/h2 [15 patients]; P<0.001) and for those without invasive aspergillosis (0.83±0.06 ng per milliliter for h1/h1 [21 patients] vs. 0.47±0.03 ng per milliliter for h2/h2 [21 patients]; P<0.001). Further supporting these findings, immunofluorescence analysis of lung-biopsy specimens from patients with invasive aspergillosis revealed decreased positivity for PTX3 in lung tissue from transplant recipients with h2/h2 donors (4 patients) as compared with transplant recipients with h1/h1 donors (5 patients) (Fig. 2B).

We performed in vitro assessment of mRNA and protein expression in purified peripheral-blood neutrophils, one major cell type expressing PTX3. Mature neutrophils do not contain detectable levels of *PTX3* mRNA.[7] In contrast, neutrophil precursors (most notably promyelocytes, as well as myelocytes plus metamyelocytes) express *PTX3* transcript.[7] The h2/h2 haplotype was correlated with lower levels of *PTX3* mRNA in h2/h2 precursors than in h1/h1 precursors (3 donors for each haplotype) (Fig. 2C). Analysis of intracellular protein levels revealed that PTX3 was constitutively present in resting neutrophils, although protein levels were markedly lower in h2/h2 neutrophils (in 14 patients) than in h1/h1 neutrophils (in 12 patients) (Fig. 2D). Defective expression of intracellular PTX3 in h2/h2 neutrophils as compared with h1/h1 neutrophils (8 patients for each haplotype) was confirmed by means of Western blotting (Fig. 2E) and immunofluorescence (Fig. 2F).

ANTIFUNGAL CAPACITY OF NEUTROPHILS AND THE H2/H2 HAPLOTYPE

To ascertain the functional consequences of the h2/h2 haplotype on antifungal effector mechanisms, we investigated the effector capacity of neutrophils bearing distinct haplotypes. Neutrophils from h2/h2 patients showed a significantly impaired ability to phagocytose conidia of *A. fumig-*



atus, with a 40-percentage-point reduction, as compared with h1/h1 cells (Fig. 3A). Moreover, h2/h2 neutrophils also showed a 20-percentage-point decrease in their conidiocidal activity in general, as compared with h1/h1 cells (Fig. 3B). Since *PTX3* haplotype block 3 underlies two distinct forms of PTX3 on the basis of the D48A substitution (48D and 48A), we assessed whether the overall defective antifungal activity displayed by h2/h2 neutrophils was linked to an altered ability of either form to bind conidia. However, both forms efficiently bound to the fungus, as assessed with the use of fluorescence-labeled PTX3 (Fig. 3C).

Given that exogenous PTX3 restores antifungal effector mechanisms in PTX3-deficient cells,[3,8] we analyzed whether the addition of PTX3 reversed the antifungal effector deficiencies observed in h2/h2 neutrophils. The addition of either form of PTX3 significantly enhanced phagocytosis of conidia by h2/h2 neutrophils (63% for 48D, and 57% for 48A) and h1/h1 neu-

Figure 2 (facing page). Effect of the h2/h2 Haplotype on PTX3 Expression.

Panel A shows PTX3 levels in samples of bronchoalveolar-lavage fluid (BAL) from patients with proven or probable invasive aspergillosis (IA) and those without IA whose donors had h1/h1 (A-C/A-C) (14 patients with IA and 21 without IA) or h2/h2 (G-A/G-A) (15 patients with IA and 21 without IA). Shown are median (±SD) levels (horizontal lines within the boxes) and interquartile ranges (lines above and below the boxes), assessed in duplicate. P values were calculated with the use of the two-tailed Mann–Whitney rank-sum test. Panel B shows immunofluorescence staining of lung-biopsy specimens with antihuman PTX3 as the primary antibody and antirabbit tetramethylrhodamine isothiocyanate as the secondary antibody. DAPI (4′,6-diamidino-2-phenylindole) was used to counterstain tissues. Images are representative of at least four samples for each haplotype assessed in two distinct preparations. Scale bars indicate 100 μm. Panel C shows analysis of *PTX3* mRNA expression in promyelocytes (PM) and myelocytes plus metamyelocytes (MY). Shown is the mean (±SD) messenger RNA (mRNA) level in three bone marrow donors for each haplotype, assessed in triplicate. Panel D shows PTX3 levels in neutrophils as assessed by enzyme-linked immunosorbent assay. Shown is the mean (±SD) protein level in at least 12 patients for each donor haplotype, assessed in duplicate. In Panels C and D, P values were calculated with the use of an unpaired Student's t-test with Bonferroni's adjustment. Panel E shows Western blot analysis of PTX3 expression in neutrophils. Blots of cell lysates were incubated with a polyclonal antibody against PTX3, and normalization was performed with an antihuman β-tubulin antibody. Panel F shows immunofluorescence imaging of PTX3 in neutrophils. Neutrophils were incubated with a primary antibody against PTX3, followed by a secondary fluorescein isothiocyanate (FITC)–conjugated antirabbit IgG antibody. Nuclei were counterstained with DAPI. Scale bars indicate 100 μm. The findings in Panels E and F are representative of those in at least eight patients tested for each haplotype combination, assessed in triplicate.

trophils (80% for 48D and 73% for 48A) (Fig. 3D). The phagocytic activity of h2/h2 neutrophils was further improved on preopsonization of conidia with either form of PTX3, since these cells were able to internalize conidia even more efficiently (82% for 48D, and 80% for 48A). Similarly, the conidiocidal activity of h2/h2 neutrophils was restored to 62% by the direct addition of 48D and to 50% by the direct addition of 48A, as well as to 60% after preopsonization of conidia with 48D and to 57% after preopsonization of conidia with 48A (Fig. 3E).

DISCUSSION

In this study, we found an association between donor h2/h2 haplotype in *PTX3* and invasive aspergillosis in patients undergoing HSCT, a finding that supports the nonredundant role of PTX3 in host defense against *A. fumigatus*.[3,4,7] More important, the increased risk of infection was observed regardless of the HLA-matching status of the donor, T-cell manipulation, and acute GVHD and prophylaxis, further sustaining a potentially independent contribution of *PTX3* variants to the development and outcome of invasive aspergillosis in the context of various strategies for modulating lymphocyte function.

PTX3 SNPs have been shown to modify the risk of pulmonary tuberculosis[23] and colonization by *Pseudomonas aeruginosa* in patients with cystic fibrosis.[24] Furthermore, these SNPs were found to alter blood levels of the PTX3 protein in Ghanaian women[25] and in lung-transplant recipients with primary graft dysfunction.[26] In our study, the h2/h2 haplotype was consistently associated with a defect in PTX3 expression in bronchoalveolar-lavage fluid, lung-biopsy specimens, and innate immune cells. This association was not confirmed systemically, a finding that suggests a requirement for the presence of PTX3 on the infected lung, where it opsonizes the fungus and helps to resolve infection.

Although neutropenia is an important risk factor for opportunistic fungal infections, most cases of aspergillosis in patients who have undergone allogeneic HSCT occur a few months after transplantation, when neutrophil levels have returned to the normal range, suggesting that neutrophils, which express PTX3 and are required for resistance to *A. fumigatus*,[7] may be particularly relevant for the observed susceptibility phenotype. Indeed, *PTX3* SNPs were not associated with invasive aspergillosis in patients with prolonged neutropenia who did not undergo transplantation, supporting the importance of the PTX3 defect in neutrophils. Because PTX3 has a role as an opsonin, limiting the amount of PTX3 available to bind the fungus most likely restrains the efficiency of phagocytosis and fungal clearance, as was observed for h2/h2 neutrophils. The exogenous addition of PTX3 to PTX3-deficient neutrophils reversed the functional deficit, confirming that the innate antifungal mechanisms

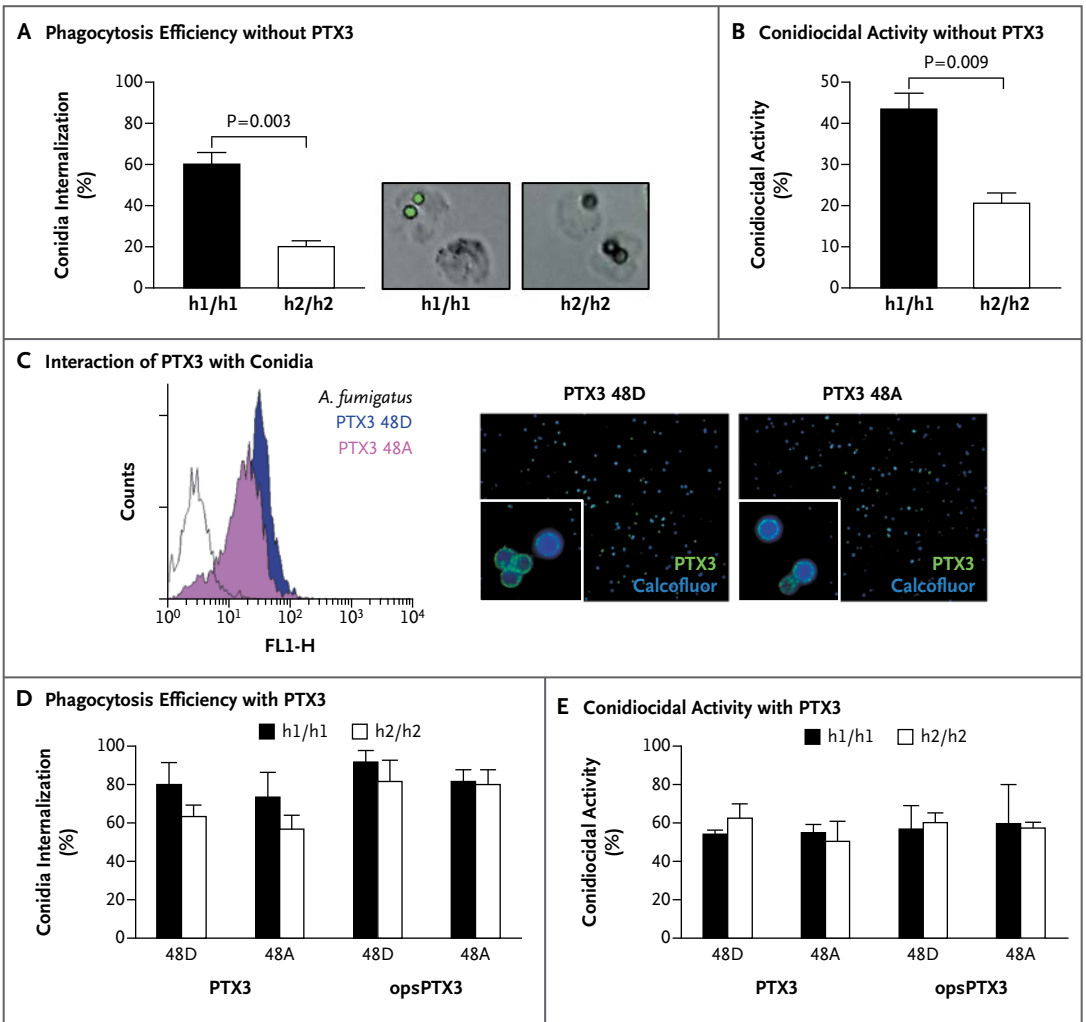


Figure 3. Antifungal Activity of Neutrophils According to the *PTX3* h2/h2 Haplotype.

Neutrophils were isolated from peripheral-blood specimens from transplant recipients whose donors had *PTX3* haplotypes h1/h1 (A-C/A-C) or h2/h2 (G-A/G-A). For the data shown in Panels A, B, D, and E, neutrophils were cultured in the presence of *Aspergillus fumigatus* conidia at a cell:fungus ratio of 1:1. Where indicated, data were obtained with the use of recombinant forms of human PTX3 bearing either an aspartic acid (48D) or an alanine (48A) at amino acid position 48. Panel A shows phagocytosis efficiency (expressed as a percentage of conidia internalization) of unopsonized conidia of *A. fumigatus*. Phagocytosis was quantified with the use of fluorescence microscopy of ingested FITC-labeled conidia. Shown are representative microscopic images. Panel B shows conidiocidal activity of neutrophils (expressed as a percentage of colony-forming-unit [CFU] inhibition) on unopsonized conidia of *A. fumigatus*. Panel C shows the interaction of PTX3 48D and 48A with viable conidia of *A. fumigatus*. Binding was assessed by analysis of Alexa Fluor 488 fluorescence by means of a fluorescence-activated cell sorter and fluorescence microscopy. Fluorescence microscopy was performed on conidia stained with calcofluor white (a chitin-specific dye) and incubated with Alexa Fluor 488–labeled PTX3. Representative histograms and images are shown. Panel D shows phagocytosis efficiency (expressed as a percentage of conidia internalization) and Panel E shows conidiocidal activity (expressed as a percentage of CFU inhibition) in the presence of PTX3 48D and 48A or conidia preopsonized with 48D and 48A (opsPTX3). In Panels A, B, D and E, mean (±SD) values are shown for at least 12 different patients for each haplotype, assessed in triplicate. P values were calculated with the use of an unpaired Student's t-test with Bonferroni's adjustment.

of these cells are compromised by a lack of sufficient PTX3 to effectively counter the fungus. Our observation that both 48A and 48D forms of PTX3 bound conidia efficiently further supports a deficiency of PTX3 in quantity, not in quality. It is also possible that PTX3 deficiency adversely affects antifungal mechanisms mediated by other innate immune receptors[27] or leukocyte recruitment in the lungs.[28]

Whatever the mechanism (or mechanisms), the absence of an association between genetic variants in transplant recipients and invasive aspergillosis suggests that epithelial PTX3 has a smaller role, if any, in the immune response to the fungus. PTX3 expression was not detectable in epithelial cells stimulated with *A. fumigatus* conidia,[3] even though its induction by proinflammatory stimuli in human lung epithelial cells has been documented.[29] Exogenous Ptx3 has been found to drive innate antifungal resistance of epithelial cells through defensins and enhancement of fungal phagocytosis,[8] suggesting that myeloid PTX3 may nonetheless play an important part in regulating epithelial antifungal mechanisms. It will be important to determine whether PTX3 deficiency also affects the initiation and skewing of adaptive immune responses to the fungus, as shown in Ptx3-deficient murine dendritic cells.[3]

Mechanistically, the decreased expression of *PTX3* transcript in neutrophil precursors is consistent with the predicted alterations in the mRNA secondary structure, suggesting that the +734A/C nucleotide change most likely affects regulation of *PTX3* expression. Also, a contribution of the intronic SNPs from the h2/h2 haplotype or other SNPs in the 5′ region to the observed phenotype cannot be ruled out. However, even though no alterations in PTX3 structure were identified as a consequence of the D48A substitution, damage to its electrostatic potential or interactions with other proteins also cannot be ruled out. The fact that the adjacent positions 47 and 49 encode cysteine residues involved in the formation of PTX3 protein complex octamers[30] supports this possibility.

In conclusion, we found that genetic deficiency of PTX3 affects the antifungal function of neutrophils. This deficiency may increase susceptibility to invasive aspergillosis in patients undergoing HSCT.

Supported by grants from the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) (to Dr. Carvalho); the German Ministry for Education and Science (03Z2JN21, to Dr. Kurzai); the European Commission (FP7-HEALTH-2009-260338, to Dr. Romani; FP7-HEALTH-2011-280873, to Dr. Mantovani), the European Research Council (ERC-2008-AdG-233417, to Dr. Mantovani; ERC-2011-AdG-293714, to Dr. Romani), Associazione Italiana per la Ricerca sul Cancro (99629, to Dr. Mantovani); and Fundação para a Ciência e Tecnologia, Portugal (SFRH/BPD/46292/2008, to Dr. Carvalho; SFRH/BD/65962/2009, to Dr. Cunha; and SFRH/BPD/70783/2010, to Dr. Almeida).

Disclosure forms provided by the authors are available with the full text of this article at NEJM.org.

We thank Rhian Gwilliam (LGC Genomics) for advice on SNP selection and assay design.

APPENDIX

The authors' affiliations are as follows: Departments of Experimental Medicine and Biochemical Sciences (C.C., A.V., L.R., A.C.) and Clinical and Experimental Medicine (A.S.B.), University of Perugia, Perugia, the Department of Clinical and Experimental Medicine, University of Parma, Parma (F.A.), Trapianto di Cellule Staminali–Ematologia 2 (A. Busca) and S.C. Microbiologia (A. Barbui), A.O. Città della Salute e della Scienza, Torino, Department of Inflammation and Immunology, Humanitas Clinical and Research Center, Rozzano (A.I., E.B., A.M.), Department of Translational Medicine, University of Milan, Milan (A.M.), Department of Medical and Surgical Sciences, University of Modena and Reggio Emilia, AOU Policlinico, Modena (L. Potenza, M.L.), Istituto di Ematologia, Università Cattolica del S. Cuore, Rome (M.C., L. Pagano), and Sigma-tau Industrie Farmaceutiche Riunite, Pomezia (G.S.) — all in Italy; Hospital de Santa Maria, University of Lisbon, Lisbon (J.F.L., P.S.S.), Institute for Molecular and Cell Biology, Universidade do Porto, Porto (B.A.), Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Braga (F.R., A.C.), and ICVS/3B's–PT Government Associate Laboratory, Braga/Guimarães (F.R., A.C.) — all in Portugal; Septomics Research Center, Friedrich-Schiller-University and Leibniz-Institute for Natural Products Research and Infection Biology, Hans-Knoell-Institute, Jena (O.K.), Department of Hematology and Oncology, University of Regensburg, Regensburg (M.G.), and Medizinische Klinik und Poliklinik II, Universitätsklinikum Würzburg, Würzburg (J.L.) — all in Germany; and the Department of Hematology, Universitaire Ziekenhuizen Leuven, Campus Gasthuisberg, Leuven, Belgium (J.A.M.).

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