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Infection and Immunity

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Polymorphisms in host immunity modulating genes and risk of invasive aspergillosis: Results from the aspBIOmics consortium

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Running title: Immune-modulating gene polymorphisms and IA

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57 **AUTHOR'S CONTRIBUTIONS**
58 MJ and JS conceived the study and participated in its design and coordination. CBL performed
59 the genetic analyses. CBL, LMC, JSC, AO-C, AC and JS performed in vitro analyses. LA-F,
60 AC, JSp, ML, ACo, CO, RR, MC-E, CS, MAL-N, AF-M, CC, TV, LF, JMA, LP, EL-F, LPo, MLu,
61 CL-F, JL, HE, LV, PCRAGA Study Group coordinated patient's recruitment and provided the
62 clinical data. JS analysed the data. MJ and JS drafted the manuscript. All authors read and
63 approved the final version of the manuscript.

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80 supplementary tables)

81 **ABSTRACT**

82 Recent studies suggest that immune-modulating single nucleotide polymorphisms (SNPs)

83 influence the risk of developing cancer-related infections. Here, we evaluated whether 36 SNPs

84 within 14 immune-related genes are associated with the risk of Invasive Aspergillosis (IA) and

85 whether genotyping of these variants might improve disease risk prediction. We conducted a

86 case-control association study of 781 immunocompromised patients, 149 of whom were

87 diagnosed with IA. Association analysis showed that the $IL4R_{rs2107356}$ and $IL8_{rs2227307}$ SNPs were

88 associated with an increased risk of IA (OR=1.92, 95%CI: 1.20-3.09 and OR=1.73, 1.06-2.81)

89 whereas the $IL12B_{rs3212227}$ and $IFN\gamma_{rs2069705}$ variants were significantly associated with a

90 decreased risk of developing the infection (OR=0.60, 0.38-0.96 and OR=0.63, 0.41-0.97). An

91 allogeneic hematopoietic stem cell transplantation (allo-HSCT)-stratified analysis revealed that

92 the effect observed for the $IL4R_{rs2107356}$ and $IFN\gamma_{rs2069705}$ SNPs was stronger in allo-HSCT

93 (OR=5.63, 1.20-3.09 and OR=0.24, 0.10-0.59) than in non-HSCT patients, suggesting that the

94 presence of these SNPs may render patients more vulnerable to infection especially under

95 severe and prolonged immunosuppressive conditions. Importantly, *in vitro* studies revealed that

96 carriers of the $IFN\gamma_{rs2069705C}$ allele showed a significantly increased macrophage-mediated

97 neutralisation of fungal conidia (*P*=0.0003) and, under stimulation conditions, produced higher

98 levels of $IFN\gamma$mRNA (*P*=0.049) and IFNγ and TNFα cytokines ($P_{LPS-96h}$=0.057, $P_{PHA-96h}$=0.036

99 and $P_{LPS+PHA-96h}$=0.030 and $P_{PHA-72h}$=0.045, $P_{LPS+PHA-72h}$=0.018, $P_{LPS-96h}$=0.058 and $P_{LPS+PHA-}$

100 $_{96h}$=0.0058, respectively). Finally, we also observed that the addition of SNPs significantly

101 associated with IA to a model including clinical variables led to a substantial improvement in the

102 discriminatory ability to predict the disease (AUC=0.659 vs. AUC=0.564, P_{LR}=$5.2 \cdot 10^{-4}$ and

103 $P_{50.000Perm}$=$9.34 \cdot 10^{-5}$). These findings suggest that the $IFN\gamma_{rs2069705}$ SNP influences the risk of IA

104 and that predictive models built with $IFN\gamma$, $IL8$, $IL12p70$ and $VEGF\alpha$ variants might be used to

105 predict disease risk and to implement risk-adapted prophylaxis or diagnostic strategies.

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109 Keywords: Invasive Aspergillosis, infection, genetic susceptibility, immuno-modulating genes.

110 **INTRODUCTION**

111 Invasive Aspergillosis (IA) is a life-threatening infection caused by *Aspergillus spp.* that affects

112 acute myelogenous leukemia (AML) and allogeneic hematopoietic stem cell transplantation (allo-

113 HSCT) patients (1-3). Despite recent improvements in the prophylaxis and treatment of IA, its

114 incidence and attributable mortality rates remain unacceptably high even among those

115 individuals who lack established risk factors (4, 5).

116

117 The initial immune response against fungal pathogens such as *Aspergillus fumigatus* the

118 principal pathogenic species, mainly relies on phagocytes, endothelial and epithelial cells that

119 recognize this fungal pathogen through pattern recognition receptors (PRRs) thus leading to

120 phagocytosis, antigen presentation and production of specific cytokines and chemokines (6, 7).

121 There are different families of PRRs including C-type lectin receptors (CLRs), Toll-like receptors

122 (TLRs), RIG-I-like receptors (RLRs) and NOD-like receptors (NLRs) that, in response to

123 *Aspergillus* pathogen-associated molecular patterns (PAMPs), activate Th_1-, Th_2- and Th_{17}-

124 related signalling cascades on phagocytes and non-professional immune cells (8-12). These

125 intracellular molecular pathways culminate in the production of both pro-inflammatory (13-18)

126 and anti-inflammatory cytokines (19, 20) and certain chemokines and their receptors (21-23) as

127 well as in the release of certain proangiogenic factors such as *VEGFA* and *bFGF* (17, 24), which

128 are also key determinants in the immune response against *Aspergillus spp*.

129

130 Although both innate and adaptive immune responses against *A. fumigatus* have been

131 extensively characterized (25, 26), it remains unclear why some immunocompromised subjects

132 develop invasive or disseminated fungal infections while others in similar clinical conditions do

133 not. The remarkable genetic variation of immune genes suggests that the presence of specific

134 genetic variants in these genes may influence their biological function and, consequently, impact

135 on the risk of developing invasive fungal infections, such as IA. In support of this hypothesis,

136 recent studies on genetic susceptibility have successfully identified several genetic variants on

137 PRR genes (*DC-SIGN, Dectin-1*, *TLRs, PTX3 and MBL*) (27-40), cytokines (*IL1 gene cluster,*

4

138   *IL10, IL12* and *IFNγ*) (32, 41-44), chemokines (*CXCL10*) (45) and immune receptors (*TNFR1*

139   and *TNFR2*) (46, 47) as factors influencing the risk of developing IA. With this background, the

140   purpose of this study was to comprehensively assess whether the presence of single nucleotide

141   polymorphisms (SNPs) within 14 immune-modulating genes (*IL4, IL4R, IL8, IL8RA, IL8RB, IL10,*

142   *IL12A, IL12B, IL13, IFNγ, IFNγR2, CCR5, MIF and VEGF*) may influence the risk of developing

143   IA. We also decided to evaluate the functional role of key variants in modulating immune

144   responses and whether selected polymorphisms could be used to predict the disease risk.

145

146   **MATERIAL AND METHODS**

147   *Study design and study population*

148   Here, we analysed whether 36 SNPs within 14 immune-modulating genes were associated with

149   IA. SNPs selection was based on three criteria: (1) SNPs within immunoregulatory genes that

150   may affect immune responses, (2) SNPs having laboratory evidence of a biological function

151   and/or (3) SNPs previously reported as associated with infectious diseases (Table 1). SNPs

152   were genotyped using KASPar® assays (LGC Genomics KBioscience, London, UK) as

153   previously described in detail (28). Patient inclusion criteria were either undergoing allo-HSCT or

154   being diagnosed with acute myeloid leukaemia (AML) or acute lymphoid leukaemia (ALL) and

155   receiving intensive remission-induction chemotherapy. A total of five hundred and ninety-three

156   patients were recruited between February 2010 and March 2014 through the aspBIOmics

157   consortium (www.aspbiomics.eu) and through two Spanish medical institutions (University

158   Hospital of Salamanca and Clinic University Hospital of Valencia) and a Spanish multicentre

159   clinical trial (PCRAGA, EU clinical trial number: 2010-019406-17) (48). Based on microbiological

160   and clinical data, one hundred and thirteen patients were diagnosed with proven or probable IA

161   according to the revised EORTC/MSG criteria (2008) (49). In order to further confirm significant

162   associations identified in our population, we extended the analysis to a second patient group

163   consisting of 188 high-risk patients (36 IA patients and 152 without IA; Table 2) recruited from

164   two Italian medical institutions (Università Cattolica del S. Cuore, Rome; and University of

165   Modena and Reggio Emilia, AOU Policlinico, Modena) and from the Virgen de las Nieves

166   University hospital (Granada, Spain) between January 2013 and January 2015. This ambitious

167   study design provided a population of 781 high-risk patients, 149 of whom were diagnosed with

168   proven and probable IA (19 proven and 130 probable IA). To our knowledge, this is one of the

169   largest population recruited so far exploring genetic susceptibility to IA. The study was approved

170   by the ethical review boards of each participating institution.

171

172   *Statistical analysis*

173   The Hardy-Weinberg Equilibrium (HWE) tests were performed in the uninfected control group by

174   a standard observed-expected chi-square (χ^2) test. Logistic regression analyses adjusted for

175   age, gender, country of origin, allo-HSCT and receipt or non-receipt of anti-fungal prophylaxis

176   were performed to determine significant associations with IA risk. SNPtool (50) and Haploview

177   were used for linkage disequilibrium (LD) blocks reconstruction and haplotype association

178   statistics. Block structures were determined according to the method of Gabriel *et al.* (51). In

179   order to account for multiple testing, we calculated an adjusted significance level using the Meff

180   method (52), which consider the number of independent marker loci (M_{effLi}=31), and the number

181   of models of inheritance tested (co-dominant, dominant, recessive and log-additive). Detailed

182   information about this method of multiple testing correction is freely available online at

183   http://neurogenetics.qimrberghofer.edu.au/SNPSpDlite. Thus, the resulting threshold for the

184   main effect analysis was 0.0004 ([0.05/31]/4).

185

186   *Cell isolation and differentiation*

187   Peripheral blood mononuclear cells (PBMCs) and monocytes were isolated from whole blood

188   collected from healthy donors after obtaining written informed consent (PI12/02688 and SECVS

189   014/2015 protocols). PBMCs were isolated by gradient centrifugation using Ficoll-Paque PLUS

190   (GE Healthcare Bio-Sciences) and monocytes were isolated by immunomagnetic selection of

191   CD_{14}^+ cells (Miltenyi Biotec). Purity of the obtained CD_{14}^+ population was assessed by

192   fluorescence-activated cell sorting analysis. Monocytes were then plated at a density of 5×10^5

193   cells/mL in 24-well plates, cultivated for 7 days in complete RPMI 1640 medium supplemented

194   with human serum and 20 ng/mL of GM-CSF to allow differentiation into macrophages. The

195   culture medium was replaced every 3 days. Genotyping of significant SNPs was performed and

196   either PBMCs or monocytes were grouped according to the genotype of interest.

197

198   *Assessment of fungicidal activity*

199   Human monocyte-derived macrophages were infected with conidia from *Aspergillus fumigatus* at

200   an effector-to-target ratio of 1:10. To measure the fungicidal ability, macrophages were allowed

201   to kill the ingested conidia for 2 h. Serial dilutions of macrophage lysates were plated on solid

202   growth media and following a 2-day incubation, the number of colony-forming units (CFU) was

203   enumerated and the percentage of CFU inhibition was calculated. In order to avoid a bias due to

204   differences in internalization rates, the supernatants collected after the co-culture were plated

205   and compared among different donors.

206

207   *IL12p70 and IFNγ stimulation assays.*

208   *IL12p70* and *IFNγ* stimulation assays were performed in PBMCs from healthy donors according

209   to a previously reported protocol (53). PBMCs were selected according to the *IL12B*$_{rs3212227}$ and

210   *IFNγ*$_{rs2069705}$ genotypes and were cultured in 2 ml of culture medium RPMI-1640 supplemented

211   with 10% sterile heat-inactivated fetal bovine serum (FBS) and an antibiotic mixture containing

212   penicillin, streptomycin and neomycin (Gibco®/Life Technologies) at 37ºC in 5% CO_2. PBMCs

213   from healthy subjects harbouring the *IFNγ*$_{rs2069705T/T}$ (n=8), *IFNγ*$_{rs2069705C/T}$ (n=8) and *IFNγ*$_{rs2069705C/C}$

214   (n=3) genotypes were incubated for 72h and 96h with phytohaemagglutinin (PHA, 2μg/ml) alone

215   or in combination with lypopolysaccharide (LPS, 100ng/ml) and IFNγ, IL12p70, TNF and IL8

216   levels were determined in triplicate using the Procartaplex, Multiplex Immunoassay

217   (Affymetrix/eBioscience) according to manufacturer's recommendations. In parallel, PBMCs

218   bearing the *IL12B*$_{rs3212227A/A}$ (n=13), *IL12B*$_{rs3212227A/C}$ (n=3) and *IL12B*$_{rs3212227C/C}$ (n=1) genotypes

219   were treated for 24h and 48h with zymosan (5μg/ml) alone or in combination with LPS

220   (100ng/ml) and correlation of cytokine levels with the *IL12B*$_{rs3212227}$ or *IL8*$_{rs321227}$ SNPs was also

7

221   analysed. After the incubation period, supernatants were collected and stored at -80ºC until

222   cytokine measurement.

223

224   *Analysis of IL4R and IFNγ mRNA expression*

225   We measured IL4R and *IFNγ* mRNA gene expression in blood samples collected from healthy

226   blood donors but also in monocyte-derived macrophages at baseline and after the stimulation

227   with conidia from *A. fumigatus* at an effector-to-target ratio of 1:2 for 8 h. Total RNA from blood or

228   monocyte-derived macrophages was extracted using RNeasy Mini Kit (QIAGEN) and reverse

229   transcribed with the iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's

230   instructions. Real-time RT-PCR was performed in an Applied Biosystems 7500HT Fast using

231   Taqman probe-based gene expression technology (Life Technologies) according to the

232   manufacturer's instructions. Statistical significance in gene expression changes was determined

233   by unpaired t test with Whelch's correction (assuming unequal variance between groups).

234

235   *Analysis of IL4R protein expression on T- and B-lymphocytes and monocytes by flow cytometry*

236   *IL4R* protein levels were determined in PBMCs carrying the wild-type *IL4R*$_{rs2107356G/G}$ (n=13),

237   heterozygote *IL4R*$_{rs2107356A/G}$ (n=24) or mutant *IL4R*$_{rs2107356A/A}$ genotypes (n=6) by flow cytometry

238   following a slightly modified version of a previously reported protocol (54). Briefly, PBMCs (1 x

239   10^6) were pre-incubated with PBS supplemented with 5% FBS + 2 mM EDTA for 10 minutes to

240   block Fc receptors. Subsequently, cells were stained for 45 minutes at ambient temperature with

241   mouse anti-human antibodies to determine the levels of *IL4R* protein expression on CD_3^+T-cells,

242   CD_{19}^+B-cells and CD_{14}^+ monocytes. The negative control consisted of cells incubated with a

243   mouse PE-IgG1 Kappa (BD Pharmingen). The analysis was performed according to the flow

244   cytometric cell surface staining method and the following antibodies were used: phycoerythrin

245   (PE)-conjugated CD_{124}^+, Peridin chlorophyll protein (PerCP)-conjugated CD_{14}^+, allophycocyanin

246   (APC)-eFluor®780-conjugated CD_3^+ and fluorescein isothiocyanate (FITC)–conjugated CD_{19}^+

247   antibodies (BD Pharmingen). Cells were acquired on a BD FACSVerse flow cytometer (BD

248   Biosciences) and the data were analysed using the FlowJo software (TreeStar Inc.). Median

249 fluorescence intensity (MFI) of the positive population was recorded for each cell type and

250 statistical differences were evaluated using an unpaired t-test with Welch's correction (two-tailed

251 P value).

252

253 *Predictive models and discriminative accuracy*

254 The value of immune-modulating polymorphisms for prediction of IA was examined using

255 stepwise logistic regression analysis. A prediction model was built that included age, gender,

256 allo-HSCT and anti-fungal prophylactic status, and those genetic variants that showed significant

257 associations with IA in the single-SNP analysis ($P<0.05$; phases 1+2). Then, using p-values as a

258 selection criterion, variables with the highest p-value were dropped and analyses were finalized

259 when all variables reached statistical significance ($P<0.05$). A predictive model with a similar

260 number of "non-significant SNPs" ($P>0.10$) was also built. The area under the curve (AUC) of a

261 receiver operating characteristic (ROC) curve analysis was used to assess the discriminative

262 accuracy of each particular model compared with a reference model including only demographic

263 and clinical variables as covariates (age, gender, allo-HSCT and anti-fungal prophylaxis status).

264 A -2 log likelihood ratio (LR) test was used to determine whether predictive models including

265 genetic information were statistically different when compared with the reference model. Finally,

266 we run a randomization test to confirm whether the improved predictive ability of the model

267 including genetic variants significantly associated with IA was consistent after 50,000 iterations.

268 Further details are included in supplementary material. All analyses were performed using R

269 (http://www.r-project.org/).

270

271 **RESULTS**

272 A total of 781 patients were enrolled in this case-control study and, among them, one hundred

273 and forty-nine were diagnosed with proven or probable IA according to the revised EORTC/MSG

274 definitions. The remaining 632 patients showed no evidence for proven or probable IA. Baseline

275 and clinical characteristics of IA and non-IA patient groups are summarized in Table 2. Overall, IA

276 and non-IA patients had a similar mean age (52.67 vs. 52.79, *P*=0.935) but IA patients showed a

9

277 significantly higher male to female gender ratio when compared with those patients with no

278 evidence of IA (1.81 vs. 1.19, P=0.031). In addition, the percentage of patients diagnosed with

279 ALL was significantly higher in IA compared with non-IA patients (16.11% vs. 8.23%, P=0.006)

280 whereas the percentage of patients with AML was significantly lower in IA compared to non-IA

281 cases (67.11% vs. 76.74%, P=0.020). Interestingly, we also observed a significantly lower

282 proportion of IA cases among those receiving posaconazole prophylaxis (P=0.017).

283

284 Thirty-six genetic variants within 14 immune-modulating genes were initially genotyped in 593

285 high-risk patients (113 IA and 480 non-IA patients). Logistic regression analysis revealed that

286 patients carrying the $IL4R_{rs2107356A/A}$ and the $VEGFA_{rs2146323A}$ and $VEGFA_{rs6900017T}$ alleles had a

287 significantly increased risk of IA (OR=2.05, 95%CI 1.24-3.40; OR=1.63, 95%CI 1.02-2.61 and

288 OR=1.76, 95%CI 1.02-3.03, respectively) whereas patients carrying the $IL12B_{rs3212227C}$ and

289 $IFN\gamma_{rs2069705C}$ alleles showed a significantly decreased risk of developing the disease (OR=0.57,

290 95%CI 0.35-0.93 and OR=0.56, 95%CI 0.36-0.88, respectively; Table 3). When a log-additive

291 model was assumed, we also found a significant association between the $VEGFA_{rs2146323}$ and

292 $VEGFA_{rs6900017}$ SNPs and an increased risk of IA (per-allele OR=1.45, 95%CI 1.04-2.03 and per-

293 allele OR=1.73, 95%CI 1.08-2.77) and a statistically significant association of the $IFN\gamma_{rs2069705}$

294 SNP with a decreased risk of developing IA (per-allele OR=0.69, 95%CI 0.49-0.97).

295

296 In order to further confirm these significant associations, the study cohort was extended by

297 recruiting 188 additional patients, 36 of whom were diagnosed with proven or probable IA. Given

298 the low number of proven and probable IA cases, we could not consider this second population

299 as an independent population for replication. An overall association analysis including 781

300 patients (149 IA and 632 non-IA patients) confirmed that carriers of the $IL4R_{rs2107356A/A}$ and

301 $IL8_{rs2227307G/G}$ genotypes had a significantly increased risk of IA when compared with those

302 carrying the wild type allele (OR=1.92, 95%CI 1.20-3.09 and OR=1.73, 95%CI 1.06-2.81)

303 whereas those subjects harbouring the $IL12B_{rs3212227C}$ and $IFN\gamma_{rs2069705C}$ alleles showed a

304 decreased risk of developing the infection (OR=0.60, 95%CI 0.38-0.96 and OR=0.63, 95%CI

10

305   0.41-0.97, respectively). When we tested the allele-dose effect of significant SNPs, we found

306   that the $IL12B_{rs3212227}$ polymorphism was significantly associated with a reduced risk of getting

307   the infection (per-allele OR=0.67, 95%CI 0.45-0.99; Table 3). As part of these association

308   analyses, we also performed haplotype analysis that confirmed that none of these

309   polymorphisms were part of risk haplotypes. We only observed a significant association with IA

310   for a relatively rare $IFN\gamma_{TC}$ haplotype whose effect was likely due to the $IFN\gamma_{rs2069705}$ SNP

311   (OR=0.34, 95%CI 0.13-0.88; Supplementary Table 1).

312

313   Interestingly, a logistic regression analysis restricted to allo-HSCT patients and considering only

314   donor genotypes and episodes of IA that occurred after transplantation (n=171) also showed that

315   the effect of the $IL4R_{2107356}$ and $IFN\gamma_{rs2069705}$ SNPs on the risk of IA was considerably stronger in

316   allo-HSCT patients compared to those patients who did not undergo transplantation (OR=5.63,

317   95%CI 1.98-16.05 vs. OR=1.48, 95%CI 0.81-2.71 and OR=0.24, 95%CI 0.10-0.59 vs. OR=0.86,

318   95%CI 0.52-1.45; respectively; Table 4). In this allo-HSCT-stratified analysis, we also found that

319   allo-HSCT patients carrying the $VEGFA_{rs3024994T}$ allele showed an increased risk of IA when

320   compared with those allo-HSCT patients carrying the wild type genotype/allele (OR=4.48, 95%CI

321   1.25-16.08; Table 4).

322

323   Although none of the reported overall, haplotype and allo-HSCT-stratified associations remained

324   significant after correction for multiple testing ($P_{Meff\_correction}$=0.0004), the association of

325    $IL4R_{rs2107356}$ and $IFN\gamma_{rs2069705}$ polymorphisms showed a marginal level of significance in allo-

326   HSCT patients when a recessive and dominant model were respectively assumed (P_{REC}=0.0009

327   and P_{DOM}=0.0011, respectively). Considering these results and those showing a suggestive

328   association between $IL12B_{rs3212227}$ SNP and risk of IA, we decided to evaluate whether the

329    $IL4R_{rs2107356}$, $IL8_{rs2227307,}$ $IL12B_{rs3212227}$ and $IFN\gamma_{rs2069705}$ variants could have a functional effect in

330   modulating the strength of immune responses against specific *Aspergillus* antigens and/or

331   stimulatory molecules. For that purpose, we first investigated whether $IFN\gamma_{rs2069705}$ and

332    $IL4R_{rs2107356}$ but also $IL12B_{rs3212227}$ and $IL8_{rs2227307}$ variants correlated with the ability of monocyte-

11

333 derived macrophages to efficiently kill fungal conidia. Interestingly, we found that macrophages

334 from donors carrying the *IFNγ*rs2069705C allele showed a significantly increased capability to kill

335 fungal spores that those from subjects carrying the wild type genotype (TT vs. TC, *P*=0.0043; TT

336 vs CC, *P*=0.0012; and TT vs. TC+CC, P=0.0003; Figure 1A). No differences in killing ability were

337 observed in macrophages from donors carrying the *IL4R*rs2107356A/A and *IL8*rs2227307G/G genotypes

338 or *IL12B*rs3212227C allele in comparison with their respective wild type allele/genotype (Figure 1B-

339 D).

340

341 Motivated by these results, we also decided to investigate whether the presence of the above-

342 mentioned SNPs correlated with cytokine levels after stimulation of PBMCs from healthy donors

343 with fungal antigens (zymosan) or stimulatory molecules (LPS and PHA). These *in vitro*

344 stimulation experiments revealed that carriers of the *IFNγ*rs2069705C allele showed an increased

345 production of IFNγ after 4 days of incubation with LPS or PHA and when both stimulating

346 reagents were used in combination (P_{LPS}=0.057, P_{PHA}=0.036 and $P_{LPS+PHA}$=0.030; Figure 2A and

347 Supplementary Table 2). We also observed that donors carrying the *IFNγ*rs2069705C allele showed

348 a drastic increase in the production of TNF at almost all time points when compared with those

349 bearing the wild type genotype ($P_{PHA-72h}$=0.045, $P_{LPS+PHA-72h}$=0.018, $P_{LPS-96h}$=0.058, and $P_{LPS+PHA-}$

350 $_{96h}$=0.0058; Figure 2B and Supplementary Table 2). In addition, we observed that subjects

351 carrying the *IFNγ*rs2069705C allele tended to have an increased production of IL12p70 when

352 compared with those carrying the wild type genotype (Figure 2C and Supplementary Table 2).

353 No correlation between *IL12p70 and IL8* levels and *IL12B*rs3212227 and *IL8*rs2227307 genotypes was

354 found. These findings suggest that the *IFNγ*rs2069705 SNP might contribute to modulate the risk of

355 IA likely through the regulation of IFNγ mRNA levels.

356

357 In order to test this hypothesis, we measured *IFNγ*mRNA expression in PBMCs from healthy

358 donors (n=21) that were grouped according to the *IFNγ*rs2069705 genotype. Importantly, we found

359 that carriers of the *IFNγ*rs2069705C allele (C/T+CC) showed a significantly increased level of *IFNγ*

360 mRNA when compared with those carrying the wild type genotype (40.85±11.65 vs. 13.87±5.43,

361 *P*=0.049; Figure 3A and 3B). Although this result pointed toward a role of this SNP in modulating

362 *IFNγ* gene expression in PBMCs, we decided to further confirm this result by looking at the

363 publicly available blood expression quantitative trait loci (eQTL) browser

364 (http://genenetwork.nl/bloodeqtlbrowser/). Of note, we found that, in agreement with our gene

365 expression data, this variant located in the promoter region of the gene (but also those

366 neighboring SNPs within the same linkage disequilibrium block) showed a positive correlation

367 with *IFNγ* mRNA expression level that ranged between P=1.01•10^{-3} and P=1.70•10^{-3}

368 (Supplementary Figure 1).

369

370 Next, we analysed the correlation between the *IL4R*$_{rs2107356}$ SNP and *IL4R* mRNA expression

371 levels in whole blood samples collected from healthy donors (n=43) and in monocyte-derived

372 macrophages at baseline and after *in vitro* stimulation with *A. fumigatus* conidia (n=12). Gene

373 expression data in blood samples did not show differences in IL4R mRNA expression levels

374 between homozygotes (GG vs. AA, *P*=0.304) but they showed a significantly increased level of

375 expression in heterozygotes when were compared either with wild type or mutant homozygotes

376 (AG vs. GG, *P*=0.0045 and AG vs. AA, *P*=0.031, respectively; Figure 3C). When carriers of the

377 *IL4R*$_{rs2107356AA}$ genotype were compared with those carrying the wild type allele (according to the

378 genetic model used in our genetic analysis), we failed to find statistical differences in IL4R

379 mRNA expression levels ($P_{AAvs.GG+AG}$=0.2937; Figure 3D). These findings did not support our

380 hypothesis suggesting a functional effect for this promoter polymorphism on mRNA expression

381 but were in agreement with those data from the blood eQTL browser that reported no

382 association of this promoter variant with IL4R mRNA expression levels in PBMCs. In line with

383 these results, we neither found any correlation between the *IL4R*$_{rs2107356}$ variant and IL4R mRNA

384 expression levels in monocytes-derived macrophages at baseline and after stimulation with *A.*

385 *fumigatus* conidia (Figure 3E and 3F). The lack of correlation between this promoter

386 polymorphism and IL4R protein level was also confirmed by flow cytometry analysis in different

387 immune cell types (CD$_{19}^{+}$, CD$_{14}^{+}$ and CD$_{3}^{+}$, cells; Figure 3G). We only found a significantly

388 increased level of IL4R in CD_{19}^{+} cells in heterozygotes when compared with those subjects

389 carrying the AA genotype (*P*=0.032; Figure 3G), which was in line with our gene expression data

390 in blood samples from healthy donors and confirmed that the effect attributed to this promoter

391 variant cannot be explained by changes in mRNA or protein expression levels. Given that *IL4R*

392 is internalized in a time-dependent manner after stimulation with *IL4*, we could not confidently

393 measure the correlation of the *IL4R*$_{2107356}$ SNP and *IL4R* protein levels under stimulation

394 conditions (data not shown).

395

396 Finally, considering the association of some of the immune-modulating SNPs with the risk of

397 developing IA and given the correlation of some of these genetic markers with cytokine levels,

398 we tested the capacity of these variants to predict with confidence the disease risk. We

399 assessed the predictive capacity of a model built with "significant SNPs" and demographic and

400 clinical variables in comparison with a reference model including only demographic and clinical

401 variables. Despite the modest population size, we found that a predictive model including 4

402 variants associated with IA, age, gender, allo-HSCT and antifungal prophylaxis status showed

403 an improvement in discriminatory ability to predict the disease when compared with the

404 reference model (AUC=0.659, 95%IC 0.596-0.722, *P*=0.000005 vs. AUC=0.564, 95%IC 0.499-

405 0.630, *P*=0.064; $P_{-2\_log\_likehood\_ratio\_test}$=0.00052; Table 5 and Figure 4). Importantly, we also

406 observed that a model built with a similar number of "non-significant SNPs", together with

407 demographic and clinical variables, did not show any significant change in predictive capacity

408 when compared with the reference model (Supplementary Table 3), which confirmed the utility of

409 significant SNPs in predicting IA. The consistency of the predictive results was supported

410 through a 50.000 permutation test that showed that none of the 50.000 permuted models

411 showed a better prediction capacity than our genetic model built with SNPs significantly

412 associated with IA ($AUC_{sort-average}$=0.6001, $SD_{sort-AUC}$=0.0158, Z score=3.7361 and P_{Z_score-}

413 $_{value\_(50.000perm)}$=9.34•10^{-05}; Table 5 and Supplementary material).

414

415 **DISCUSSION**

14

416   In this study, we report an association between genetic variants within *IL4R*, *IL8*, *IL12B* and *IFN*γ

417   genes and the risk of developing IA. Carriers of the *IL4R*rs2107356A/A and *IL8*rs2227307G/G genotypes

418   had a significantly increased risk of developing the infection whereas patients carrying the

419   *IL12B*rs3212227C and *IFN*γrs2069705C alleles showed a substantially decreased risk of IA when

420   compared with those harbouring the wild type allele/genotype. Although none of these

421   associations persisted after a restrictive correction for multiple testing, we found that the

422   association of the *IL4R*rs2107356 and *IFN*γrs2069705 polymorphisms reached marginal significance in

423   the allo-HSCT patient population, which pointed towards an impact of these variants in

424   modulating the risk of developing IA, particularly in high-risk populations. Based on these results

425   but also those suggesting overall associations of polymorphisms within the *IL4R*, *IL8*, *IL12B* and

426   *IFN*γ genes at the conventional significance threshold of *P*≤0.05, it seems plausible to suggest

427   that polymorphisms within these genes may affect gene function and therefore contribute to the

428   pathogenesis of IA. We hypothesized that both *IL4R*rs2107356 and *IFN*γrs2069705 polymorphisms,

429   located in the promoter region of their respective genes, may affect gene expression and,

430   consequently, have an effect in modulating *IL4*- and *IFN*γ-mediated immune responses against

431   *Aspergillus*. Likewise, we hypothesized that *IL8*rs2227307 and *IL12B*rs3212227 intronic polymorphisms

432   might affect alternative splicing of *IL8* and *IL12B* mRNA and even alter mRNA expression

433   thereby dysregulating *IL8*- and *IL12*-mediated Th1 immune responses against fungal pathogens.

434   In line with these arguments, a number of previous studies have reported associations of these

435   polymorphisms with immune-related diseases (55, 56) and infections (57-60) including IA (45).

436   In particular, we confirmed an association previously reported by Mezger *et al.* (2008) between

437   the *IFN*γrs2069705 SNP and the risk of IA (45). This supports our hypothesis that this association is

438   likely to be true and that this variant may play a role in modulating the immune response against

439   *Aspergillus*.

440

441   Recent studies in humans and animal models have demonstrated that *IL4/IL4R*, *IL8*, *IL12* and

442   *IFN*γ have a central role in IA (19, 61-65). In particular, *IFN*γ seems to be a key factor as its

443   enhanced production (14, 66) or its therapeutic administration (63), boosts the production of free

444   oxygen radicals and the neutrophil-mediated damage of fungal hyphae and promotes resistance

445   to the infection (19, 67). It is also well documented that the production of *IL8 and IL12p70* by

446   epithelial and dendritic cells in response to conidia enhances Th$_1$-mediated immune responses

447   (61, 68, 69) and increases resistance to IA (62) whereas its neutralization produces a marked

448   increase in susceptibility to IA (15, 19). IL12p70 also mediates enhanced cytotoxic activity of NK

449   cells and CD$_8^+$-T cells and promotes the secretion of *IFNγ* by CD$_4^+$-T cells, which is an essential

450   process for an efficient clearance of inhaled *Aspergillus fumigatus* spores. Conversely, *IL4*

451   secretion activates Th$_2$-CD$_4^+$-T cell immune responses and leads to a significant decrease in Th$_1$

452   immune responses and, consequently, increases susceptibility to *Aspergillus* infection (19, 70).

453   Similarly, it has also been demonstrated that the lack of *IL4* cytokine increases Th$_1$ immune

454   responses characterized by high production of *IL12* and an enhanced *IL12*-mediated production

455   of *IFNγ* by T lymphocytes thereby leading to an increase in resistance to developing the infection

456   (19).

457

458   In light of these results and in order to better characterize the role of *IFNγ*, *IL4R*, *IL8* and *IL12B*

459   polymorphisms in modulating immune responses against fungal antigens and/or specific

460   stimulatory molecules, we proceeded to perform functional assays in PBMCs and monocyte-

461   derived macrophages from healthy donors. Importantly, we found that subjects carrying the

462   *IFNγ*$_{rs2069705C}$ allele showed an increased ability to kill *A. fumigatus* conidia than those carrying

463   the wild type allele. This important finding support our genetic findings but also those from a

464   previous study (45) suggesting that *IFNγ*$_{rs2069705}$ promoter variant might play a key role in

465   modulating the strength of immune responses against *Aspergillus* likely through the modulation

466   on IFNγ mRNA expression. Importantly, we also observed that, under stimulating conditions,

467   PBMCs from carriers of the *IFNγ*$_{rs2069705C}$ allele showed an increased production of IFNγ and TNF

468   cytokines than those individuals carrying the wild type allele. In addition to this, we found that the

469   *IFNγ*$_{rs2069705}$ SNP correlated with *IFNγ* mRNA levels in PBMCs from healthy donors, which again

16

470   suggested that this polymorphism or another causative polymorphism in strong linkage

471   disequilibrium with it may be involved not only in the control of *IFNγ* production but also in the

472   subsequent induction of *TNF* production. Taking all these findings together, we propose a central

473   role for this variant in determining the risk of IA in allo-HSCT and in leukaemia patients

474   undergoing intensive chemotherapy.

475

476   Although genetic data suggested that polymorphisms within IL4R, IL8 and IL12B influence the

477   risk of developing IA, functional experiments did not show any correlation between these SNPs

478   and their respective mRNA and/or protein levels. Therefore, we suggest that these variants

479   might exert their biological function by modulating other biological processes such as mRNA

480   processing (splicing or turnover) or mRNA stability or even act at post-transcriptional level.

481   Further studies are now warranted to replicate our findings and to experimentally identify the

482   functional role of these SNPs in determining the risk of IA.

483

484   Finally, given the genetic and/or functional effect observed for variants within *IFNγ*, *IL4R*, *IL8*,

485   *IL12B* and *VEGFA* genes, we found also interesting to determine the impact of these variants in

486   predicting the disease risk. We found that a model built with $IL8_{rs2227307}$, $IL12B_{rs3212227}$, $IFN\gamma_{rs2069705}$

487   and $VEGFA_{rs6900017}$ SNPs showed a significantly improved discriminatory ability to predict the

488   disease when it was compared with a model that included demographic and clinical variables.

489   Importantly, when a similar number of "non-significant SNPs" were added to the reference

490   model, we did not observe any significant change in predictive capacity, which confirmed that

491   only a model built with these significant SNPs could have capacity to predict the infection. In

492   support of this finding, we also observed that the AUC of this model was systematically higher

493   than the AUC observed for 50.000 iterative models, which emphasizes the importance of

494   considering predictive models to assist in the clinical decision-making process and to improve in

495   novel strategies to prevent IA occurrence.

496

497 This study has both strengths and limitations. Study strengths include a multicentre population-

498 based design, a relatively large sample size, and the high number of genetic polymorphisms

499 analysed. This allowed us for the first time to perform predictive analyses to assess the potential

500 utility of genetic variants in predicting with confidence the risk of developing IA. Potential

501 weaknesses include limited antifungal prophylaxis data availability for a subset of patients and a

502 relatively low number of proven or probable IA cases that limited the study's statistical power to

503 rule out spurious associations. To minimize this limitation, the most relevant associations were

504 functionally validated.

505

506 In conclusion, our data suggest that immune-modulating polymorphisms have an impact on the

507 risk of IA and that genotyping of these variants could help to predict the risk of IA and therefore

508 be useful to establish a risk-adapted anti-fungal prophylaxis strategy.

509

514

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777 **TABLE AND FIGURE LEGENDS**

778 **Table 1.** Selected SNPs within immune-modulating genes.
779
780   Abbreviations: SNP, single nucleotide polymorphisms, UTR, untranslated region. References included in this
781   table are listed in the supplementary material.
782
783 **Table 2.** Baseline and clinical characteristic of IA and non-IA patient groups.
784
785   Abbreviations: HSCT: Hematopoietic stem cell transplantation, AML: acute myeloid leukemia, ALL: acute
786   lymphoid leukemia; UHS, University hospital of Salamanca (Spain); GHV, General hospital of Valencia (Spain);
787   PCRAGA clinical trial (EU clinical trial number: 2010-019406-17); CSC, Università Cattolica del S. Cuore, Rome
788   (Italy); MO, University of Modena and Reggio Emilia, Modena (Italy). P≤0.05 was considered significant.
789   * Some patients had several prophylactic drugs.
790   † Prophylaxis status was only available in 99 subjects (15 IA and 72 non-IA patients).
791   ∂ Percentage calculated according to the number of patients with prophylaxis data available.
792
793 **Table 3.** Association of polymorphisms within immunoregulatory genes and invasive
794 aspergillosis.
795
796   Abbreviations: OR, odds ratio; CI, confidence interval. Abbreviations: n/s, not specified; SNP, single nucleotide
797   polymorphism; UTR, untranslated region. Estimates were adjusted for age, sex, country of origin, allo-SCT, and
798   prophylaxis status (ever use of prophylaxis). P<0.05 in bold.
799
800   [a]Discovery population: aspBIOmics+PCRAGA+Valencia+Salamanca populations; N=593 hematological patients
801   [b]Overall: n=781; after extension with 188 high-risk patients [39 HSCT and 149 non-HSCT patients]; 87 of them
802   with prophylaxis data.
803   [†]Estimates according a recessive model of inheritance.
804   [§]SNP signifcantly associated with IA according a log-additive model of inheritance.
805
806   [§]$IFNG_{rs2069705}$ (per-allele OR= 0.69, 95%CI 0.49-0.97; P_{trend}=0.032).
807   [§]$VEGFA_{rs2146323}$ (per-allele OR= 1.45, 95%CI 1.04-2.03; P_{trend} =0.029).
808   [§]$VEGFA_{rs6900017}$ (per-allele OR= 1.73, 95%CI 1.08-2.77; P_{trend}=0.027).
809   [§]$IL12B_{rs3212227}$ (per-allele OR= 0.67, 95%CI 0.45-0.99; P_{trend} =0.040).
810
811
812 **Table 4.** Association of immunoregulatory SNPs and IA in allo-HSCT patients (n=171).
813
814   Abbreviations: OR, odds ratio; CI, confidence interval. Abbreviations: n/s, not specified; SNP, single nucleotide
815   polymorphism; UTR, untranslated region. Estimates were adjusted for age, sex, country of origin, severe
816   neutropenia, and prophylactic status (ever having prophylaxis). P<0.05 in bold.
817
818   [a]Overall population (Discovery population+extension).
819   [†]Estimates according a recessive model of inheritance.
820   [§]SNP associated with IA infection according to a log-additive model of inheritance.
821   *Estimates calculated according a co-dominant model (homozygotes for the rare allele were not found).
822
823   [§]$IL4R_{rs2107356}$ (per-allele OR= 2.17, 95%CI 1.18-3.98; P_{trend}=0.0097).
824   [§]$IFNG_{rs2069705}$ (per-allele OR= 0.50, 95%CI 0.26-0.95; P_{trend}=0.027).
825   [§]$VEGFA_{rs3024994}$ (per-allele OR= 3.19, 95%CI 1.08-9.45; P_{trend}=0.033).
826
827 **Table 5**. Discriminative value *AUC* for models including immune-modulating variants.
828
829   [a]Including age, gender, allo-HSCT and prophylactic status as variables never dropped from models.
830   **$IL4R_{rs2107356}$ and $VEGFA_{rs2146323}$ polymorphisms were not significant and were dropped from the model.
831   Residual deviance (Reference model): 433.21
832   Residual deviance (Significant SNPs model): 413.31
833   After removing missing values, 455 subjects (85 IA and 370 non-IA cases) were available for prediction capacity
834   analysis.
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841 **Figure 1.** Fungicidal activity of monocyte-derived macrophage according to *IFNγ*[A], *IL12B* [B],
842 *IL4R* [C] and *IL8* [D] genotypes.
843
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845 **Figure 2.** *IFNγ, TNFα* and *IL12p70* cytokine levels on stimulated PBMCs according to the
846 *IFNγ*$_{rs2069705}$ genotypes.
847
848 PBMCs from healthy donors were stimulated with Zymosan (5µg/ml) and PHA (2 µg/ml) alone or in combination
849 with LPS (100 ng/ml). Supernatants were harvested for *IFNγ*, *TNFα* and *IL12p70* analysis at 72 and 96h.
850
851
852 **Figure 3.** *IFNγ* [A and B] and *IL4R* mRNA [C-F] and protein [G] expression levels according to
853 the *IL4R*$_{rs2107356}$ genotypes.
854
855 Correlation between *IFNγ*$_{rs2069705}$ genotypes and *IFNγ* mRNA expression levels were analysed in PBMCs from
856 healthy donors at baseline or stimulated with *Aspergillus* conidia [A and B]. Correlation between *IL4R*$_{rs2107356}$
857 genotypes and IL4R mRNA expression levels were analysed in blood samples from healthy donors [C and D] and
858 in monocyte-derived macrophages at baseline or stimulated with *Aspergillus* conidia [E and F]. Correlation
859 between this promoter variant and IL4R protein levels were also analysed in different immune cells (CD$_{19}^+$, CD$_{14}^+$
860 and CD$_3^+$) by flow cytometry [G].
861
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863 **Figure 4.** Receiver operating characteristics (ROC) curve analysis.
864
865 ROC curves summarize the accuracy of prediction for each particular model. The model including SNPs
866 significantly associated with IA and demographic and clinical variables (marked in blue) showed a significantly
867 improved predictive capacity comparared with a reference model including only demographic and clinical variables
868 (marked in red).
869 AUC=0.659 vs. AUC=0.564.
870

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Figure 1

[A]

IFNγ$_{rs2069705}$

IFNγ$_{rs2069705}$

[D]

IL8$_{rs2227307}$

[B]

IL12B$_{rs3212227}$

[C]

IL4R$_{rs2107356}$

Figure 2

[A] **IFNG levels / IFNG genotype**

IFNG$_{rs2069705T/T}$, n=8
IFNG$_{rs2069705C/T}$, n=8
IFNG$_{rs2069705C/C}$, n=3

Legend:
- LPS+PHA-IFNG$_{rs2069705CT+CC}$ (filled circle, solid line)
- LPS+PHA-IFNG$_{rs2069705TT}$ (open circle, solid line)
- PHA-IFNG$_{rs2069705CT+CC}$ (filled triangle, dashed line)
- PHA-IFNG$_{rs2069705TT}$ (open triangle, dashed line)
- LPS-IFNG$_{rs2069705CT+CC}$ (filled square, solid line)
- LPS-IFNG$_{rs2069705TT}$ (open square, dashed line)

[B] **TNF levels / IFNG genotype**

IFNG$_{rs2069705T/T}$, n=8
IFNG$_{rs2069705C/T}$, n=8
IFNG$_{rs2069705C/C}$, n=3

Legend:
- LPS+PHA-IFNG$_{rs2069705CT+CC}$
- LPS+PHA-IFNG$_{rs2069705TT}$
- PHA-IFNG$_{rs2069705CT+CC}$
- PHA-IFNG$_{rs2069705TT}$
- LPS-IFNG$_{rs2069705CT+CC}$
- LPS-IFNG$_{rs2069705TT}$

[C] **IL12p70 levels / IFNG genotype**

IFNG$_{rs2069705T/T}$, n=8
IFNG$_{rs2069705C/T}$, n=8
IFNG$_{rs2069705C/C}$, n=3

Legend:
- LPS+PHA-IFNG$_{rs2069705CT+CC}$
- LPS+PHA-IFNG$_{rs2069705TT}$
- PHA-IFNG$_{rs2069705CT+CC}$
- PHA-IFNG$_{rs2069705TT}$
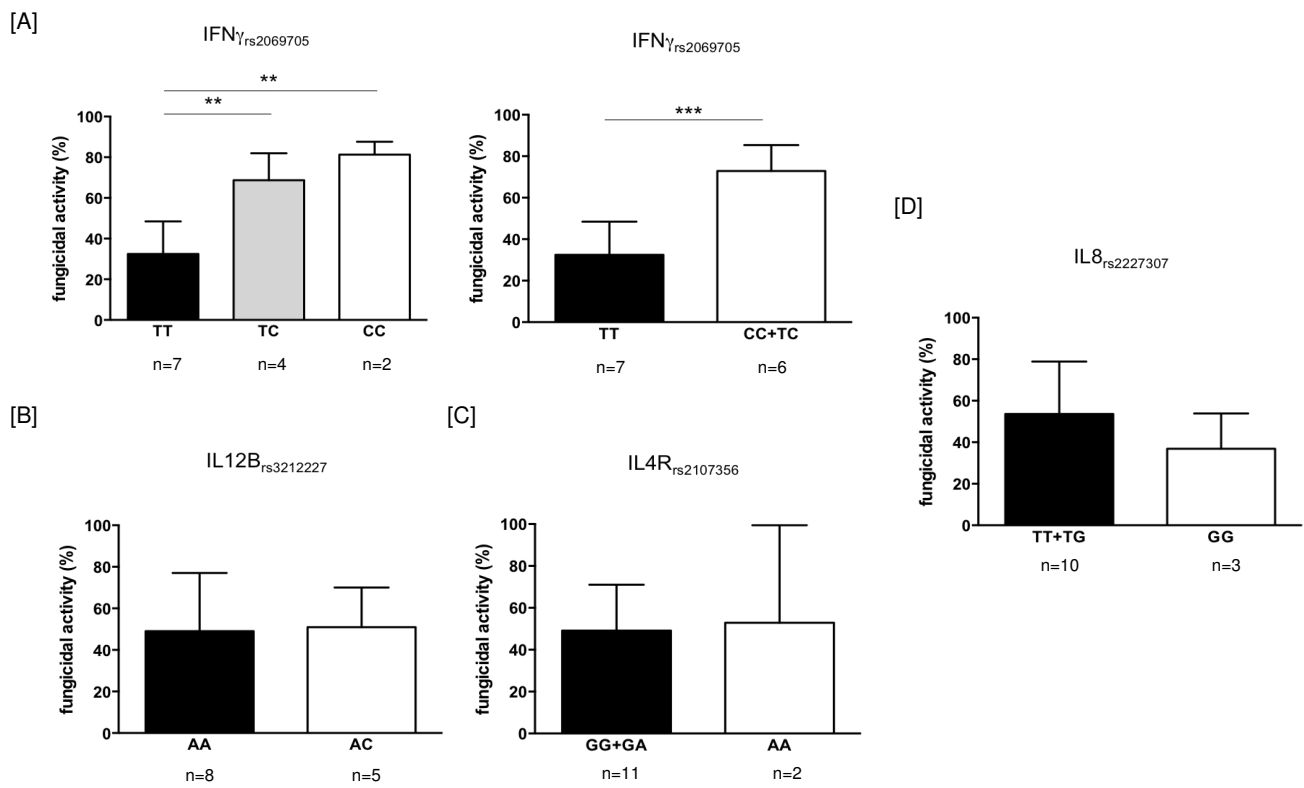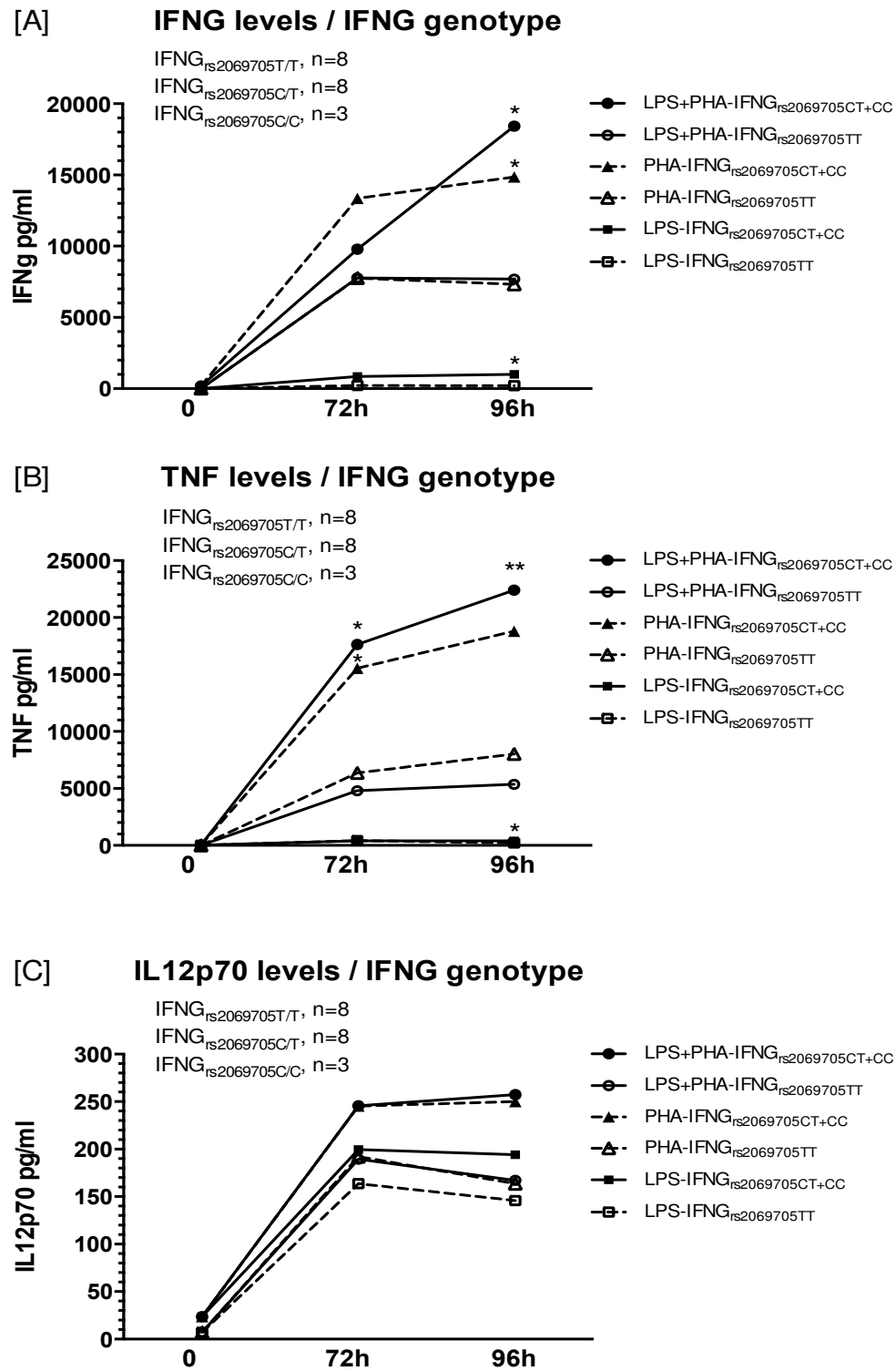- LPS-IFNG$_{rs2069705CT+CC}$
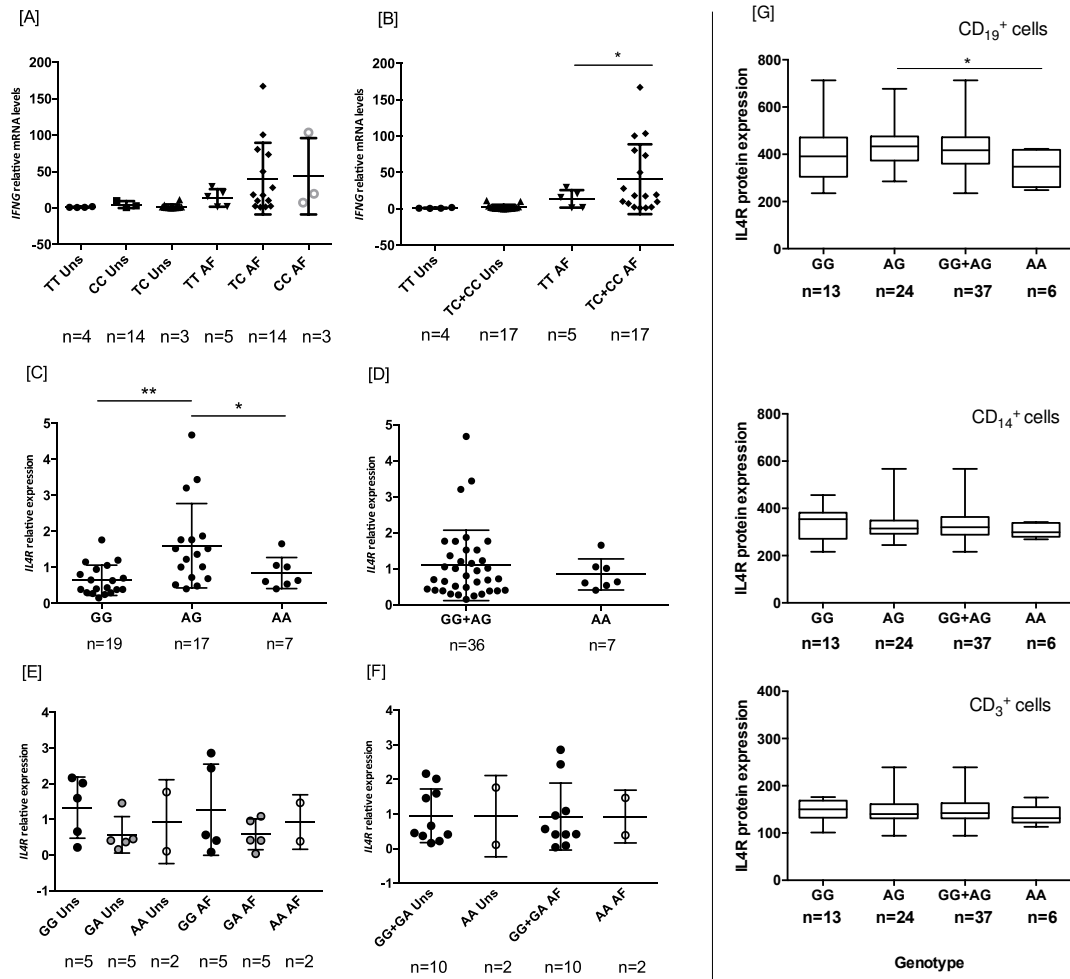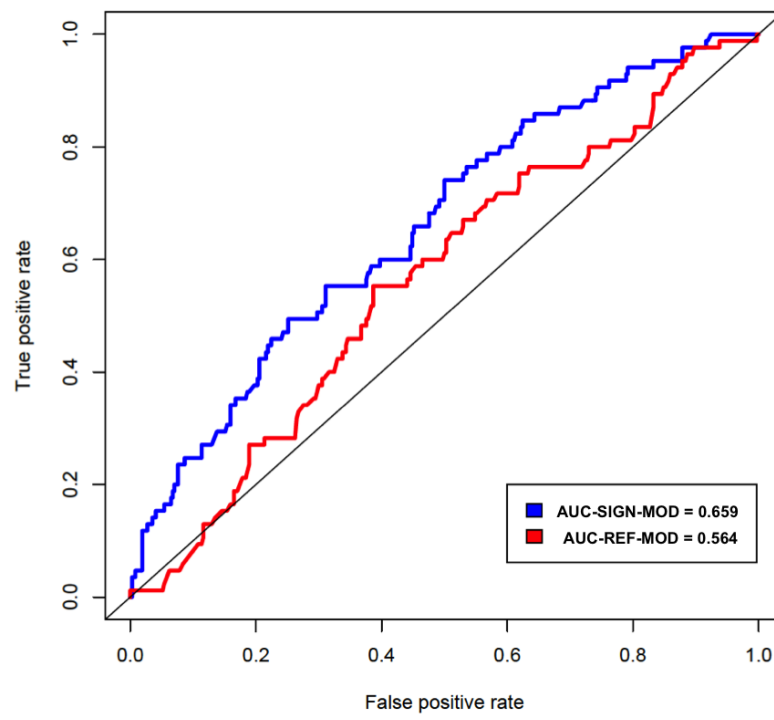- LPS-IFNG$_{rs2069705TT}$

Figure 3

Figure 4

Table 1. Selected SNPs within immune-modulating genes.

Gene name	Gene symbol_SNP	dbSNP rs#	Nucleotide substitution	Aa change/Location	Reported associations with infectious diseases / reported or potential functionality	References
Interleukin 4 (IL4)	IL4_-1098	rs2243248	G/T	Promoter	Associated with chronic disseminated candidiasis	(1)
	IL4_Ex1-168	rs2070874	C/T	Intronic	Unknown	
	IL4_IVS2-1443	rs2243268	A/C	Intronic	Unknown	
	IL4_ IVS3-9	rs2243290	A/C	Intronic	Unknown	
Interleukin 4 receptor (IL4R)	IL4R_-29429 (-3223)	rs2057768	A/G	Promoter	Associated with soluble IL4R protein levels	(2)
	IL4R_-28120 (-1914)	rs2107356	A/G	Promoter	Unknown	
	IL4R_Ex11+828	rs1801275	A/G	Q576R	Associated with enhanced responsiveness to IL4	(3, 4)
Interleukin 8 (IL8)	IL8_-251	rs4073	A/T	Promoter	Associated with increased levels of IL-8 and susceptibility to bacterial urinary tract infection, recurrent Clostridium difficile infection, AIDS, Helicobacter pylori related gastric diseases and mycetoma	(5-8)
	IL8_ IVS1+230 (+396)	rs2227307	G/T	Intronic	Associated with susceptibility to periodontitis	(9)
CXC-Chemokine receptor 1 (IL8RA)	CXCR1_Ex2+860	rs2234671	G/C	S276T	Associated with chronic HBV infection. Predicted to affect IL8 signalling (Benign; Polyphen)	(10)
CXC-Chemokine receptor 2 (IL8RB)	CXCR2_Ex3-1010	rs1126580	A/G	Intronic	Unknown	
Interleukin 10 (IL10)	IL10_IVS1-286	rs3024491	G/T	Intronic	Unknown	
	IL10_Ex5+210	rs3024496	C/T	Intronic	IL10_Ex5+210G alleles is associated with the decreased production of IL-10 by peripheral blood leukocytes in response to helminth infection	(11)
Interleukin 12 alpha (IL12A)	IL12A_IVS2-798	rs582054	A/T	Intronic	Unknown	
Interleukin 12 beta (IL12B)	IL12B_Ex8+159 (+1188)	rs3212227	A/C	Intronic	IL12B_+1188C allele is associated with an increased risk of lepromatous leprosy	(12)
Interleukin 13 (IL13)	IL13_-1069	rs1800925	C/T	Promoter	Alters the expression of IL13 and the binding of nuclear factors to the IL13 promoter. Associated with an increased risk of severe respiratory syncytial virus (RSV) infection.	(13, 14)
	IL13_Ex4+98	rs20541	C/T	R144Q	Modifies the IL13-mediated Th2 effector functions and correlates with IL13 activity and levels (114Q carriers have higher levels of IL13 compared to 144R)	(15)
	IL13_IVS3-24	rs1295686	A/G	Intronic	Unknown	
Interferon gamma (IFNγ)	IFNG_-1615	rs2069705	C/T	Promoter	Associated with a reduced risk of IA	(16)
	IFNG_IVS3+284 (+2109)	rs1861494	C/T	Intronic	Unknown	
Interferon gamma receptor 2 (IFNγR2)	IFNGR2_Ex7-128	rs1059293	C/T	Intronic	Unknown	
	IFNGR2_Ex2-16	rs9808753	A/G	Q64R	Predicted to affect IFNγ signalling (Possibly damaging; Polyphen)	
C-C chemokine receptor type 5 (CCR5)	CCR5_IVS1+246	rs1799987	A/G	Intronic	Associated with CCR5 protein levels and HIV-1	(17, 18)
	CCR5_IVS1+151	rs2734648	G/T	Intronic	Part of an haplotype associated with HIV-1	(19)

Macrophage migration inhibitory factor (MIF)	*MIF_-173*	rs755622	C/G	Promoter	MIF_-173CC is associated with pulmonary tuberculosis	(20, 21)
Vascular Endothelial Growth Factor alpha (VEGFA)	*VEGFA_-2578*	rs699947	A/C	Promoter	VEGF_-2578CC was associated with higher or lower VEGF expression. Associated with urinary tract infection.	(22-24)
	VEGFA_-7	rs25648	C/T	Promoter	Associated with higher levels of VEGFA mRNA	(25)
	VEGFA_IVS2+1378	rs3024994	C/T	Intronic	Unknown	
	VEGFA_IVS7-919	rs3025035	C/T	Intronic	Unknown	
	VEGFA_6112	rs2146323	A/C	-	Unknown	
	VEGFA_IVS-99	rs3024997	A/G	Intronic	Unknown	
	VEGFA_IVS7+763	rs3025030	C/G	Intronic	Unknown	
	VEGFA_5530	rs998584	G/T	-	Unknown	
	VEGFA_5958bp 3'of STP	rs6899540	A/C	-	Unknown	
	VEGFA_6119bp 3'of STP	rs6900017	C/T	-	Unknown	
	VEGFA_Near gene	rs6905288	A/G	-		

Abbreviations: SNP, single nucleotide polymorphisms, UTR, untranslated region. References included in this table are listed in the supplementary material.

Table 2. Baseline and clinical characteristic of patients with or without invasive aspergillosis (IA).

Phases 1	aspBIOmics consortium + UHS-GHV-PCR-AGA populations			
	overall (n=593)	IA patients (n=113)	non-IA patients (n=480)	*P* value
Demographic variables				
age (range)	51.46 ± 15.08	51.70 ± 13.20	51.40 ± 15.51	0.834
sex ratio (male/female)	1.27 (332/261)	1.90 (74/39)	1.16 (258/222)	**0.031**
Haematological disease				
AML	412 (69.48)	66 (58.41)	346 (72.08)	**0.006**
ALL	73 (12.31)	23 (20.35)	50 (10.42)	**0.006**
other	108 (18.21)	24 (21.24)	84 (17.50)	0.429
allo-HSCT	299 (50.42)	65 (57.52)	234 (48.75)	0.116
Ever received prophylaxis *				
posaconazole	73 (12.31)	10 (8.85)	63 (13.13)	0.277
itraconazole	49 (8.26)	13 (11.50)	36 (7.50)	0.230
echinocandins	30 (5.06)	2 (1.77)	28 (5.83)	0.125
voriconazole	21 (3.54)	6 (5.31)	15 (3.13)	0.397
amphotericin B	4 (0.67)	0 (0.00)	4 (0.83)	0.738
Never received prophylaxis	450 (75.89)	87 (76.99)	363 (75.63)	0.855
Phase 2	**CSC-MO + VNH population**			
	overall (n=188)	IA patients (n=36)	non-IA patients (n=152)	*P* value
Demographic variables				
age (range)	56.93 ± 18.12	55.75 ± 19.50	57.21 ± 17.84	0.665
sex ratio (male/female)	1.32 (107/81)	1.57 (22/14)	1.27 (85/67)	0.705
Haematological disease				
AML	173 (92.02)	34 (94.44)	139 (91.45)	0.799
ALL	3 (1.60)	1 (2.78)	2 (1.32)	0.912
other	12 (6.38)	1 (2.78)	11 (7.24)	0.545
allo-HSCT	39 (20.74)	3 (8.33)	36 (23.68)	0.069
Ever received prophylaxis†				
posaconazole	40 (40.40)	3 (11.11)	37 (51.39)	**0.0007**
itraconazole	7 (7.07)	3 (11.11)	4 (5.56)	0.603
echinocandins	1 (1.01)	0 (0.00)	1 (1.39)	0.608
voriconazole	2 (2.02)	0 (0.00)	2 (2.78)	0.942
amphotericin B	16 (16.16)	2 (7.41)	14 (19.44)	0.253
Never received prophylaxis∂	69 (69.70)	24 (88.89)	45 (62.50)	**0.022**
Phases 1+2	**aspBIOmics consortium + UHS-GHV-PCR-AGA + CSC-MO population**			
	overall (n=781)	IA patients (n=149)	non-IA patients (n=632)	*P* value
Demographic variables				
age (range)	52.77 ± 16.03	52.67 ± 15.00	52.79 ± 16.27	0.935
sex ratio (male/female)	1.28 (439/342)	1.81 (96/53)	1.19 (343/289)	**0.031**
Haematological disease				
AML	585 (74.90)	100 (67.11)	485 (76.74)	**0.020**
ALL	76 (09.73)	24 (16.11)	52 (08.23)	**0.006**
other	120 (15.36)	25 (16.78)	95 (15.03)	0.685
allo-HSCT	338 (43.28)	68 (45.64)	270 (42.72)	0.579
Ever received prophylaxis				
posaconazole	113 (16.33)	13 (9.29)	100 (18.12)	**0.017**
itraconazole	56 (8.09)	16 (11.43)	40 (7.25)	0.148
echinocandins	31 (4.48)	2 (1.43)	29 (5.25)	0.085
voriconazole	23 (3.32)	6 (4.29)	17 (3.08)	0.655
amphotericin B	20 (2.89)	2 (1.43)	18 (3.26)	0.383
Never received prophylaxis∂	519 (75.00)	111 (79.29)	408 (73.91)	0.229

Abbreviations: HSCT: Haematopoietic stem cell transplantation, AML: acute myeloid leukemia, ALL: acute lymphoid leukemia; UHS, University hospital of Salamanca (Spain); GHV, General hospital of Valencia (Spain); PCRAGA clinical trial (EU clinical trial number: 2010-019406-17); CSC, Università Cattolica del S. Cuore, Rome (Italy); MO, University of Modena and Reggio Emilia, Modena (Italy) and Virgen de las Nieves University hospital (VNH). P≤0.05 was considered significant.
* Some patients had several prophylactic drugs.
† Prophylaxis status was only available in 99 subjects (15 IA and 72 non-IA patients).
∂ Percentage calculated according to the number of patients with prophylaxis data available.

Table 3. Associations found between immunoregulatory polymorphisms and invasive aspergillosis.

Variant_dbSNP	Gene	OR (95% CI)[a]	P_{value}	OR (95% CI)[b]	P_{value}
rs2243248	*IL4*	1.19 (0.63-2.26)	0.59		
rs2070874	*IL4*	0.91 (0.53-1.55)	0.72		
rs2243268	*IL4*	0.85 (0.52-1.38)	0.50		
rs2243290	*IL4*	0.67 (0.39-1.16)	0.14		
rs2057768	*IL4R*	1.20 (0.75-1.92)	0.44		
rs2107356	*IL4R*	**2.05 (1.24-3.40)[†]**	**0.0063**	**1.92 (1.20-3.09)[†]**	**0.008**
rs1801275	*IL4R*	1.00 (0.63-1.59)	0.99		
rs4073	*IL8*	1.02 (0.64-1.61)	0.95		
rs2227307	*IL8*	**1.72 (1.00-2.94)[†]**	**0.049**	**1.73 (1.06-2.81)[†]**	**0.031**
rs2234671	*IL8RA*	1.57 (0.80-3.08)	0.20		
rs1126580	*IL8RB*	1.50 (0.88-2.54)	0.13		
rs3024491	*IL10*	1.09 (0.67-1.78)	0.72		
rs3024496	*IL10*	1.16 (0.71-1.90)	0.55		
rs582054	*IL12A*	1.09 (0.64-1.84)	0.76		
rs3212227	*IL12B*	**0.57 (0.35-0.93)**	**0.021**	**0.60 (0.38-0.96)[§]**	**0.029**
rs20541	*IL13*	0.76 (0.46-1.24)	0.26		
rs1800925	*IL13*	0.85 (0.54-1.36)	0.51		
rs1295686	*IL13*	0.73 (0.45-1.16)	0.18		
rs2069705	*IFNG*	**0.56 (0.36-0.88)[§]**	**0.012**	**0.63 (0.41-0.97)**	**0.035**
rs1861494	*IFNG*	0.74 (0.47-1.17)	0.20		
rs1059293	*IFNGR2*	0.98 (0.57-1.69)	0.93		
rs9808753	*IFNGR2*	1.10 (0.65-1.85)	0.72		
rs1799987	*CCR5*	1.40 (0.83-2.36)	0.20		
rs2734648	*CCR5*	1.07 (0.67-1.71)	0.76		
rs755622	*MIF*	1.38 (0.84-2.25)	0.20		
rs25648	*VEGFA*	1.11 (0.63-1.97)	0.72		
rs699947	*VEGFA*	1.28 (0.75-2.18)	0.35		
rs3024994	*VEGFA*	1.61 (0.86-3.03)	0.15		
rs3025035	*VEGFA*	1.31 (0.78-2.22)	0.31		
rs2146323	*VEGFA*	**1.63 (1.02-2.61)**	**0.040**	1.46 (0.95-2.27)	0.085
rs3024997	*VEGFA*	1.04 (0.67-1.61)	0.87		
rs3025030	*VEGFA*	1.00 (0.58-1.70)	0.99		
rs998584	*VEGFA*	0.66 (0.41-1.06)	0.088		
rs6899540	*VEGFA*	0.84 (0.51-1.40)	0.50		
rs6900017	*VEGFA*	**1.76 (1.02-3.03)[§]**	**0.046**	1.47 (0.87-2.47)	0.16
rs6905288	*VEGFA*	0.83 (0.52-1.31)	0.42		

Abbreviations: OR, odds ratio; CI, confidence interval. Abbreviations: n/s, not specified; SNP, single nucleotide polymorphism; UTR, untranslated region. Estimates were adjusted for age, sex, country of origin, allo-SCT, and prophylaxis status (ever use of prophylaxis). P<0.05 in bold. *P*<0.0004 was defined as multiple testing significance threshold.

[a]Phase 1 (aspBIOmics + PCRAGA+Valencia+Salamanca populations; N=593 hematological patients).
[b]Overall (N=781; after extension with 188 high-risk patients [39 HSCT and 149 non-HSCT patients]; 87 of them with prophylaxis data).
[†]Estimates according a recessive model of inheritance.
[§]SNP signifcantly associated with IA according a log-additive model of inheritance.

[§]*IFNG*$_{rs2069705}$ (per-allele OR= 0.69, 95%CI 0.49-0.97; P_{trend}=0.032).
[§]*VEGFA*$_{rs2146323}$ (per-allele OR= 1.45, 95%CI 1.04-2.03; P_{trend} =0.029).
[§]*VEGFA*$_{rs6900017}$ (per-allele OR= 1.73, 95%CI 1.08-2.77; P_{trend}=0.027).
[§]*IL12B*$_{rs3212227}$ (per-allele OR= 0.67, 95%CI 0.45-0.99; P_{trend} =0.040).

Table 4. Associations found between immunoregulatory SNPs and IA in allo-HSCT patients (n=171).

Variant_dbSNP	Gene	OR (95%CI)	P_{value}
rs2243248	IL4	0.93 (0.25-3.44)	0.91
rs2070874	IL4	1.40 (0.56-3.52)	0.47
rs2243268	IL4	1.15 (0.49-2.73)	0.75
rs2243290	IL4	0.93 (0.35-2.45)	0.88
rs2057768	IL4R	1.30 (0.56-3.03)	0.54
rs2107356	IL4R	**5.63 (1.98-16.05)[†§]**	**0.0009**
rs1801275	IL4R	0.50 (0.20-1.24)	0.12
rs4073	IL8	0.76 (0.33-1.73)	0.51
rs2227307	IL8	1.21 (0.51-2.86)	0.67
rs2234671	IL8RA	1.48 (0.42-5.15)	0.55
rs1126580	IL8RB	2.39 (0.92-6.20)[†]	0.072
rs3024491	IL10	1.08 (0.44-2.70)	0.86
rs3024496	IL10	0.74 (0.30-1.83)	0.52
rs582054	IL12A	1.73 (0.61-4.91)	0.29
rs3212227	IL12B	0.64 (0.26-1.57)	0.32
rs20541	IL13	0.80 (0.32-1.99)	0.63
rs1800925	IL13	1.96 (0.84-4.58)	0.12
rs1295686	IL13	0.53 (0.22-1.29)	0.16
rs2069705	IFNG	**0.24 (0.10-0.59)[§]**	**0.0011**
rs1861494	IFNG	0.63 (0.27-1.49)	0.29
rs1059293	IFNGR2	1.53 (0.59-3.97)	0.37
rs9808753	IFNGR2	0.78 (0.29-2.09)	0.62
rs1799987	CCR5	1.75 (0.65-4.69)	0.26
rs2734648	CCR5	1.04 (0.44-2.48)	0.93
rs755622	MIF	0.63 (0.25-1.61)*	0.33
rs25648	VEGFA	1.39 (0.53-3.66)	0.51
rs699947	VEGFA	0.51 (0.19-1.36)	0.18
rs3024994	VEGFA	**4.48 (1.25-16.08)[§]**	**0.022**
rs3025035	VEGFA	1.96 (0.77-4.99)	0.16
rs2146323	VEGFA	0.86 (0.38-1.97)	0.72
rs3024997	VEGFA	0.71 (0.31-1.62)	0.41
rs3025030	VEGFA	1.11 (0.41-3.01)*	0.84
rs998584	VEGFA	0.84 (0.32-2.22)	0.72
rs6899540	VEGFA	0.62 (0.23-1.69)	0.34
rs6900017	VEGFA	2.68 (0.97-7.42)	0.061
rs6905288	VEGFA	1.04 (0.43-2.53)	0.92

Abbreviations: OR, odds ratio; CI, confidence interval. Abbreviations: n/s, not specified; SNP, single nucleotide polymorphism; UTR, untranslated region. Estimates were adjusted for age, sex, country of origin, severe neutropenia, and prophylactic status (ever having prophylaxis). P<0.05 in bold. P<0.0004 was defined as corrected significance threshold.

[†]Estimates according a recessive model of inheritance.
[§]SNP associated with IA infection according to a log-additive model of inheritance.
*Estimates calculated according a co-dominant model (homozygotes for the rare allele were not found).

[§]$IL4R_{rs2107356}$ (per-allele OR= 2.17, 95%CI 1.18-3.98; P_{trend}=0.0097).
[§]$IFNG_{rs2069705}$ (per-allele OR= 0.50, 95%CI 0.26-0.95; P_{trend}=0.027).
[§]$VEGFA_{rs3024994}$ (per-allele OR= 3.19, 95%CI 1.08-9.45; P_{trend}=0.033).

Table 5. Discriminative value *AUC* for models with or without immune-modulating variants.

Reference model				
SNPs	P-value	OR 95%CI	AUC 95%CI[a]	*P*-value
Age	0.898	1.001 (0.985-1.017)		
Gender	0.033	1.721 (1.045-2.835)		
Allo-SCT	0.785	0.934 (0.570-1.529)		
Prophylactic status	0.790	1.080 (0.612-1.906)	$0.564 (0.499\text{-}0.630)^{\partial}$	0.064
Predictive model built with 4 significant SNPs*				
SNPs	P-value	OR 95%CI	AUC 95%CI[a]	*P*-value
$IL8_{rs2227307}$	0.024	1.952 (1.093-3.489)		
$IL12B_{rs3212227}$	0.016	0.508 (0.292-0.884)		
$IFNG_{rs2069705}$	0.031	0.583 (0.358-0.952)		
$VEGFA_{rs6900017}$	0.040	1.814 (1.026-3.207)		
Age	0.951	1.001 (0.984-1.018)		
Gender	0.064	1.626 (0.972-2.719)		
Allo-SCT	0.757	0.923 (0.557-1.532)		
Prophylactic status	0.525	1.210 (0.672-2.179)	$0.659 (0.596\text{-}0.722)^{\partial}$	0.000005

[a]Including age, gender, allo-SCT and prophylactic status as variables never dropped from models.

*IL4R$_{rs2107356}$ and *VEGFA*$_{rs2146323}$ polymorphisms were not significant and were dropped from the model.

∂ These models showed a statistically different prediction capacity (-2log likehood ratio test, df=4, *P*=0.00052).

Residual deviance (Reference model): 433.21

Residual deviance (Significant SNPs model): 413.31

After removing missing values, 455 subjects (85 IA and 370 non-IA cases) were available for prediction capacity analysis.

Permutation analysis: Average AUC of null distribution (50.000 models)=0.6001; $SD_{50.000AUC}$=0.0158;

Z-score_value$_{50.000perm}$=3.7361 and $P_{50.000perm}$=9.34•10^{-05}.