

## Polymorphisms in genes related to the complement system and antibody-mediated cardiac allograft rejection

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**Background.** Heart transplantation (HT) is a life-saving treatment for patients with end-stage heart failure. One of the main problems after HT is the humoral response termed antibody-mediated rejection (AMR). Complement activation plays a key role in AMR contributing to graft damage. The aim of this study was to analyze genetic variants in genes related to the complement pathways that could be associated with the development of AMR.

**Methods.** Analysis of 51 genes related to the complement pathway was performed by next-generation sequencing in 46 HT recipients, 23 with and 23 without AMR. Statistical analysis was performed with SNPstats and R.

**Results.** We identified 2 single nucleotide polymorphisms, 1 in the mannose-binding lectin 2 gene (p.Gly54Asp-*MBL2*) and 1 in the complement factor properdin gene (p.Asn428(p=)-*CFP*), that showed significant association with the absence and development of AMR, respectively. Moreover, the presence of the rare allele in p.Gly54Asp-*MBL2* control patients correlated with an immunodeficiency of mannose-binding lectin (6.24 ng/ml vs 207.50 ng/ml,  $p < 0.01$ ), whereas the presence of the rare allele p.Asn428(p=)-*CFP* in patients with AMR correlated with higher levels of properdin protein (14.65 µg/ml vs 10.77 µg/ml,  $p < 0.05$ ).

**Conclusions.** AMR is a complex phenotype affected by many recipient factors. Variants in p.Gly54Asp-*MBL2* and p.Asn428(p=)-*CFP* genes, encoding mannose-binding lectin 2 and properdin, may influence the risk of AMR.

### Keywords

Complement genes; Antibody-mediated rejection; Heart transplantation; Mannose binding lectin; Properdin

Heart transplantation (HT) is a well-established life-saving treatment for patients presenting with end-stage cardiac failure. However, it has been established that approximately 15%–25% of patients who receive a transplant experience cell-mediated rejection episodes, and some develop antibody-mediated rejection (AMR).<sup>1,2</sup> AMR in HT is associated with hemodynamic compromise, increased graft loss, cardiac allograft vasculopathy (CAV), and increased mortality.<sup>2</sup> The true incidence of AMR is unknown; however, values ranging from 3% to 85%, owing to diverse diagnostic criteria and variations in screening frequency, have been reported.<sup>3,4</sup> Our research group reported an incidence of <3% when using the criteria of allograft dysfunction and C4d deposition in endomyocardial biopsy.<sup>5</sup>

Allograft rejection caused by antibodies can be mediated by different mechanisms, as opposed to T-cell rejection. Classically, antibody induces acute rejection through the fixation and activation of the complement cascade, resulting in tissue injury.<sup>4,6</sup> Complement, which is a multicomponent system of receptors, regulators, and effector molecules, is a very powerful amplifier of the innate and adaptive immunity contributing to the pathogenesis of AMR.<sup>6,7</sup>

More than 50 genes encoding proteins of complement components, receptors, and regulators have been described.<sup>8</sup> The complement system consists of many proteins, mainly proteases, which create a cascade when triggered.<sup>9</sup> There are 3 pathways of complement activation (Figure S1, available in the online version of this article at [www.jhltonline.org](http://www.jhltonline.org)): (1) the classical pathway, which is activated by antibodies bound to antigens and forming immune complexes; (2) the lectin pathway, which is activated by carbohydrates such as mannose-binding lectin (MBL); and (3) the alternative pathway, which is activated in the absence of antibodies by spontaneous hydrolysis of C3. Each pathway converges in the formation of a C3 convertase molecule, whose function is to cleave C3.

Several polymorphisms and mutations in genes related to the complement system have been associated with different diseases, including renal, ocular, and infectious diseases.<sup>8,10–12</sup> In the field of transplantation, most studies have focused on renal transplant outcomes. For example, the GGCG haplotype and 450 C/T polymorphism in *C5* and *C5a* genes were associated with lower renal allograft function<sup>13</sup> and low risk for graft rejection,<sup>14</sup> respectively, whereas a polymorphism in *CD46* was associated with acute renal allograft rejection.<sup>15</sup> Moreover, mutations in complement genes may predict recurrence and graft outcome in renal transplant recipients with a typical hemolytic-uremic syndrome.<sup>16,17</sup> However, other studies showed no association between polymorphisms in *C3* and lectin complement pathway genes and graft outcome.<sup>18–20</sup> One limitation of these studies was that they analyzed <6 genes involved in the complement pathway.<sup>13–20</sup> Recently, Ermini et al<sup>21</sup> analyzed the relationship between single nucleotide polymorphisms (SNPs) in 47 complement genes and graft survival, serum creatinine, delayed graft function, and acute rejection in donors and recipients of renal transplants. They found 1 donor SNP, c.304+363T>C (rs4935047), in the non-coding region of the mannose-binding lectin 2 gene (*MBL2*) associated with worse graft outcome.

In HT, several genetic polymorphisms in cytokine genes have been associated with the risk of acute rejection.<sup>22</sup> Despite the important role of complement in AMR, no study to our knowledge has described the role of variants in complement genes in AMR after HT. Thus, the aim of the present study was to investigate the association between variants in complement genes and the development of AMR after HT.

## Methods

### *Patients*

A retrospective case-control study was conducted in samples from a historical cohort of patients who received a HT at Complejo Hospitalario Universitario de A Coruña (CHUAC) from June 2000 until November 2016. The study was carried out on 46 HT recipients, 23 with and 23 without AMR. The 23 AMR cases were matched to 23 control patients by sex, age ( $\pm 5$  years), and follow-up post-transplant.

The diagnostic criteria to define the cases of AMR were different according to whether the transplant date was before or after 2013. AMR in patients who underwent HT before 2013 ( $n = 15$ ) was defined according to the following criteria: (1) allograft dysfunction (left ventricular ejection fraction  $< 30\%$  and/or heart failure), (2) no evidence of other causes of allograft dysfunction (acute cellular rejection or CAV), (3) evidence of complement activation on endomyocardial biopsy (C4d and/or C3d staining), and (4) favorable response to therapy addressing AMR (e.g., plasmapheresis, rituximab, steroid boluses). AMR in patients who underwent HT after 2013 ( $n = 8$ ) was classified according to International Society for Heart and Lung Transplantation (ISHLT) categories pAMR1(I+), pAMR1(H+), pAMR2, and pAMR3<sup>23</sup> and inclusion criteria of having at least 1 positive endomyocardial biopsy (pAMR1 or higher). Control patients did not present with any distinguishing signs of AMR (pAMR0) or allograft dysfunction. Each control patient was followed a minimum of 6 months longer than the AMR event of the corresponding case. All HT recipients provided biological samples for DNA analysis.

All samples used for this work had been stored in the collection of samples for research of the advanced heart failure and cardiac transplant unit of CHUAC (“Colección de muestras para la investigación de insuficiencia cardíaca avanzada y trasplante cardíaco” registered in the Instituto de Salud Carlos III as C\_0000419, 2012/348). The study was approved by the local Ethics Committee (“Comité ético de investigación de Galicia,” reference: 2014/012) and conformed to the ethical guidelines of the Declaration of Helsinki. Written informed consent was obtained for sample collection and genetic screening tests.

### *Genetic study*

Genomic DNA was isolated from clots and blood samples using QIAamp DNA Blood Mini Kit (Qiagen GmbH, Hilden, Germany) following the manufacturer’s instructions and was stored at 4°C until use. The genetic analysis included 51 genes related to the complement pathway (Figure S1 and Table S1, available in the online version of this article at [www.jhltonline.org](http://www.jhltonline.org)). For this purpose, target resequencing was performed using TruSight One Sequencing Panel (Illumina, San Diego, CA), consisting of 4,813 genes, including the 51 genes selected. The sequence library was constructed using 50 ng of each genomic DNA, quantified with Qubit 2.0 (Life Technologies). The NextSeq500 platform (Illumina) was used to perform 151-bp paired-end sequencing according to manufacturer’s instructions. The sequencing reads were aligned to the human reference genome (hg19). The extracted variants, reported in variant call format output file, were evaluated for coverage and Qscore. In addition, coding and adjacent intronic regions of the 51 genes were visualized via Integrative Genomics Viewer to confirm the presence of the variants detected.

### *In silico tools*

The potential effect of missense SNP associated with AMR was predicted using in silico tools. Moreover, the minor allele frequency (MAF) of the SNPs detected by next-generation sequencing (NGS) was extracted from the Single Nucleotide Polymorphism Database (dbSNP) and/or the Exome Aggregation Consortium (ExAC).

### ***Localization***

Topologic placement of the mutations was done using the Swissprot database (<http://ca.expasy.org/uniprot/>) and the bibliography previously described. The Uniprot database provides generally accepted residue ranges corresponding with each domain region and specialized subregion. The Swiss-Pdb Viewer (v.4.10) was used to generate the models of the mutated protein structure by replacing the native aminoacid for the variant.

### ***Predicting damaging amino acid substitution***

Three online tools were used to predict the pathogenicity of the missense variants: SIFT ([http://sift.jcvi.org/www/SIFT\\_seq\\_submit2.html](http://sift.jcvi.org/www/SIFT_seq_submit2.html)), Polyphen-2 (<http://genetics.bwh.harvard.edu/%20pph2/>), and PhDSNP (<http://snps.uib.es/phd-snp/phdsnp.html>). The ClinVar database was also consulted.

### ***Sanger sequencing***

Direct sequencing of 15 amplicons (Table S2, available in the online version of this article at [www.jhltonline.org](http://www.jhltonline.org)), containing at least 1 variant by NGS each, was performed to evaluate sensitivity [TP/(TP+FN)], specificity [TN/(TN+FP)], and accuracy [(TP+TN)/(TP+FP+FN+TN)] of the NGS technique. In these formulas, TP means true-positive, TN means true-negative, FP means false-positive, and FN means false-negative.

### ***Enzyme-linked immunosorbent assay measurements***

Pre-transplant serum samples were used for the determination of functional MBL ( $n = 9$ , 5 samples from patients with AMR with the reference sequence in the position c.161 and 4 samples from control patients that carried the variant c.161G>A) and properdin ( $n = 16$ , 5 with AMR and variant c.1284C>T and 11 without AMR and with the reference sequence in the position c.1284) levels, using commercially available competitive sandwich enzyme-linked immunosorbent assay kits (MBL, Human, ELISA kit HK323-01, and Properdin, Human, ELISA kit HK334-01) from Hycult Biotech (Uden, Netherlands).

MBL measurable concentration range was 0.4–100 ng/ml. Serum samples were diluted 1:10 with MBL-binding buffer, and all measurements were made in duplicate. Properdin measurable concentration range was 0.3–20 ng/ml. Serum samples were diluted 1:2,000 with dilution buffer, and all measurements were made in triplicate. In both cases, the absorbance at 450 nm was measured in a spectrophotometer. The concentration of MBL varies among individuals from <5 to >5,000 ng/ml<sup>24</sup>; the properdin concentration varies from 5 to 15 µg/ml.<sup>25</sup>

### ***Statistical analyses***

Continuous variables are expressed as mean  $\pm$  SD. Discrete variables are shown as percentage. For the genetic analysis, multiple inheritance models (codominant, dominant, recessive, and log-additive) were first applied by using SNPStats software. In addition, the genetic analysis was redone using R software for SNPs with significant allele association with any of the multiple models. Differences in allele and genotype frequencies between the 2 groups were assessed by McNemar's chi-square test using R. A  $p$ -value of 0.05 was used as a significance threshold.

For analysis of serum levels, values were expressed as median and interquartile range (IQR). To assess the significance of differences of protein serum levels, the Mann-Whitney  $U$  test for non-normally distributed values was performed using R software.  $p < 0.05$  was considered statistically significant. The

R package and commands used to perform the statistical analyses are presented in the supplementary data (available in the online version of this article at [www.jhltonline.org](http://www.jhltonline.org)).

## Results

### *Baseline clinical characteristics*

Of the 46 patients studied, 86.96% were men, and the mean age of recipients at time of transplantation was 50.1 years  $\pm$  16.0 (Table 1). The mean follow-up post-transplant was 7.3 years  $\pm$  3.6, and the most common heart disease in patients requiring HT was ischemic cardiomyopathy (39.1%). Table 1 displays the study population demographics, including immunosuppressive regimens. Furthermore, available data from the characterization of anti-HLA antibodies in both patients with AMR and control patients were included (Table S3, available in the online version of this article at [www.jhltonline.org](http://www.jhltonline.org)). Using the pathologic AMR classification in patients after 2013,<sup>23</sup> 4 patients were classified as pAMR(I+), and 4 patients were classified as pAMR2.

**Table 1.** Clinical Characteristics of Patients

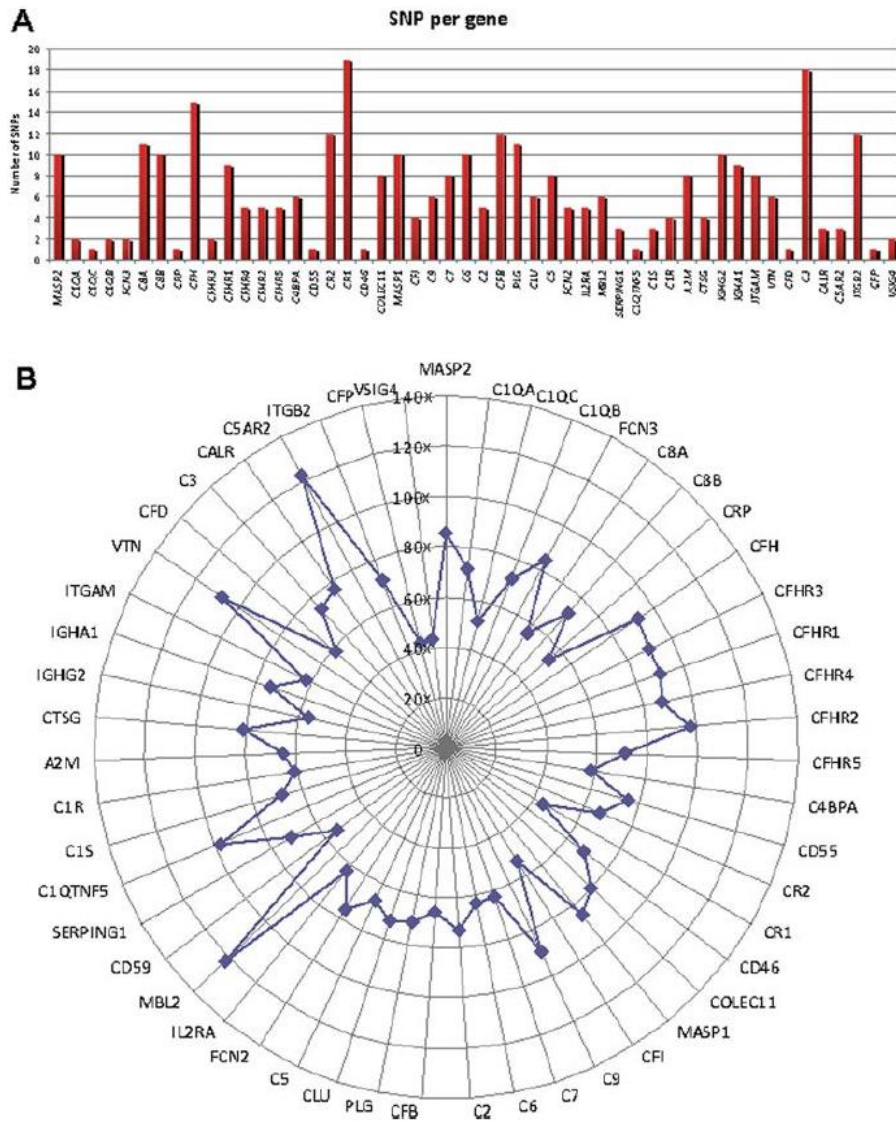
Variable	Total (n = 46 patients)	AMR (n = 23 patients)	Control (n = 23 patients)
Age, years, mean $\pm$ SD	50.1 $\pm$ 16.0	50.2 $\pm$ 16.5	50.0 $\pm$ 15.9
Male	87.0% (40)	87.0% (20)	87.0% (20)
Female	13.0% (6)	13.0% (3)	13.0% (3)
Primary heart disease			
Dilated cardiomyopathy	34.8% (16)	26.1% (6)	43.5% (10)
Ischemic cardiomyopathy	39.1% (18)	52.2% (12)	26.1% (6)
Valvular cardiomyopathy	10.9% (5)	8.7% (2)	13.0% (3)
Others	15.2% (7)	13.0% (3)	17.4% (4)
Time since transplant			
< 2 years	10.4% (5)	17.4% (4)	4.4% (1)
2–5 years	23.9% (11)	30.4% (7)	17.4% (4)
$\geq$ 6 years	65.2% (30)	52.2% (12)	78.3% (18)
Time to AMR diagnosis after transplant, years, mean $\pm$ SD		3.5 $\pm$ 0.6	
Cytomegalovirus status			
Donor +/recipient +	58.7% (27)	56.5% (13)	60.9% (14)
Donor –/recipient –	2.2% (1)	0	4.4% (1)
Donor +/recipient –	23.9% (11)	30.4% (7)	17.4% (4)
Donor –/recipient +	13.0% (6)	13.0% (3)	13.0% (3)
Unknown	2.2% (1)	0	4.4% (1)
Immunosuppression			
Cyclosporine	75.0% (36)	75.0% (18)	75.0% (18)
Plasmapheresis	32.6% (15)	65.2% (15)	0
Rituximab	32.6% (15)	65.2% (15)	0
Steroids	100% (46)	100% (23)	100% (23)
OKT3	6.5% (3)	12.5% (3)	0
Mycophenolate mofetil	91.3% (42)	87.0% (20)	95.7% (22)
Tacrolimus	56.5% (26)	65.2% (15)	47.4% (11)
Everolimus	32.6% (15)	26.1% (6)	39.1% (9)
Basiliximab	89.1% (41)	82.6% (19)	95.7% (22)
Sandimmune	14.6% (7)	20.8% (5)	8.3% (2)
Daclizumab	4.4% (2)	4.4% (1)	4.4% (1)

AMR, antibody-mediated rejection.

### ***Genotype association with AMR***

After sequencing all coding regions and the  $\pm 10$  intronic pair bases in 51 genes related to the complement pathway (Figure S1, available in the online version of this article at [www.jhltonline.org](http://www.jhltonline.org)), 319 polymorphisms in 50 genes were found (Figure 1A and Table S4, available in the online version of this article at [www.jhltonline.org](http://www.jhltonline.org)). No SNPs were found in *CD59* gene. The mean coverage overall the genes related to the complement cascade was 72.7-fold  $\pm$  2.4 (Figure 1B). To determine the accuracy, specificity, and sensitivity of the NGS protocol, we followed 42 selected amplicons containing at least 1 variant by NGS. From 8,481 readable bases in the Sanger sequencing, we observed 8,445 TN calls and 33 TP calls. No FP calls and 3 FN calls were found, together resulting in a sensitivity of 91.7%, specificity of 100%, and accuracy of 99.7%. After manual inspection of the FN calls that only had been identified by Sanger, we detected that all FN calls appeared in the *IGHA1* gene in the position chr14:106.173.808. However, in the 3 samples, Integrative Genomics Viewer allowed visualization of the variant in < 10% readings. Thus, we postulated that these FN must be related to the quality of the sequence in this position.

A genetic association study was carried out to test for an association between AMR and polymorphisms in the complement cascade genes. First, an analysis with SNPstats was performed. The results showed that, from the 319 SNPs found, 8 variants (rs1048118-*CFP*, rs1800450-*MBL2*, rs11627594-*IGHG2*, rs1407-*IGHA1*, rs1130656-*PLG*, rs1450656-*C7*, rs17611-*C5*, rs25681-*C5*) were associated with AMR in at least 1 of the multiple inheritance models applied. Second, when the association function of R was applied, all 8 SNPs previously identified were significantly associated with AMR. However, when the McNemar chi-square test was done, only 2 SNPs were significantly associated with AMR: (1) the missense variant c.161G>A, p.Gly54Asp in *MBL2* gene, rs1800450 (Table 2), and (2) the synonymous variant c.1284C>T, p.Asn428(p=) in *CFP* gene, rs1048118 (Table 3).



**Figure 1.** SNP distribution per gene and sequence coverage. (A) Bar graph showing the distribution of SNPs per gene. (B) Graph representing the sequence coverage of the genes related to the complement cascade analyzed in the study. Overall complement genes mean coverage is  $72.7 \pm 2.4$ .

**Table 2.** Statistical Analysis of Variant p.Gly54Asp (rs1800450) in *MBL2* Gene in Control and AMR Groups

rs1800450	Model	Genotype	Control	AMR	OR (95%CI)	p-value	AIC	BIC	
SNPstats	Codominant	G/G	13 (56.5%)	21 (91.3%)	1.00	0.02 <sup>a</sup>	61.7	67.2	
		G/A	9 (39.1%)	2 (8.7%)	0.1 (0.03–0.7)				
		A/A	1 (4.3%)	0 (0%)	0.00 (0.00–NA)				
	Dominant	G/G	13 (56.5%)	21 (91.3%)	1.00	0.006 <sup>a</sup>	60	63.7	
		G/A-A/A	10 (43.5%)	2 (8.7%)	0.12 (0.02–0.66)				
	Recessive	G/G-G/A	22 (95.7%)	23 (100%)	1.00	0.2	66.4	70	
		A/A	1 (4.3%)	0 (0%)	0.00 (0.00–NA)				
	Overdominant	G/G-A/A	14 (60.9%)	21 (91.3%)	1.00	0.01 <sup>a</sup>	61.5	65.2	
		G/A	9 (39.1%)	2 (8.7%)	0.2 (0.03–0.8)				
	R (F(x)association)	Log-additive	—	—	—	0.1 (0.03–0.7)	0.005 <sup>a</sup>	59.7	63.4
		Codominant	G/G	13 (56.5%)	21 (91.3%)	1.00	0.02 <sup>a</sup>	61.7	
			G/A	9 (39.1%)	2 (8.7%)	0.1 (0.03–0.7)			
A/A			1 (4.3%)	0 (0%)	0.00				
Dominant		G/G	13 (56.5%)	21 (91.3%)	1.00	0.005 <sup>a</sup>	60		
		G/A-A/A	10 (43.5%)	2 (8.7%)	0.1 (0.02–0.7)				
Recessive		G/G-G/A	22 (95.7%)	23 (100%)	1.00	1	66.4		
		A/A	1 (4.3%)	0 (0%)	0.00				
Overdominant		G/G-A/A	14 (60.9%)	21 (91.3%)	1.00	0.01 <sup>a</sup>	61.5		
		G/A	9 (39.1%)	2 (8.7%)	0.2 (0.03–0.8)				
Log-additive		—	—	—	0.1 (0.03–0.7)	0.02 <sup>a</sup>	59.7		
McNemar's test		Chi-square = 4.9 with 1 df				0.03 <sup>a</sup>			

AIC, Akaike information criterion; AMR, antibody-mediated rejection; BIC, Bayesian information criterion; CI, confidence interval; OR, odds ratio. <sup>a</sup>Statistical significance.

**Table 3.** Statistical Analysis of Variant rs1048118 in *CFP* Gene in Control and AMR Groups

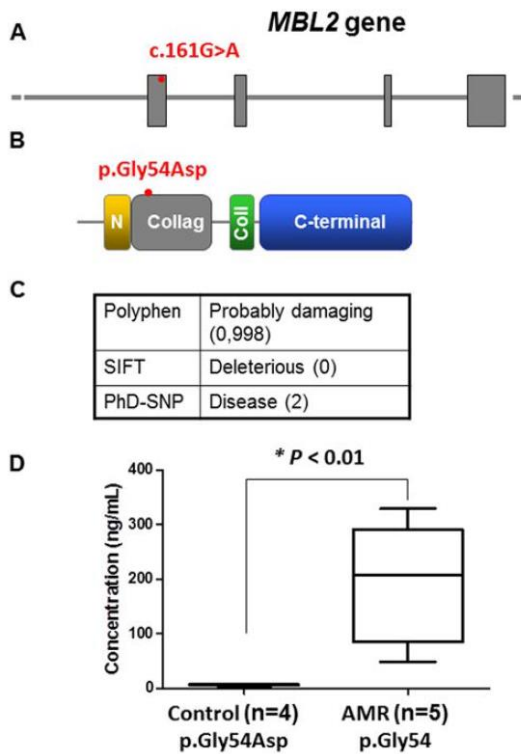
rs1048118	Model	Genotype	Control	AMR	OR(95%CI)	p-value	AIC	BIC	
SNPstats	Codominant	C/C	21 (91.3%)	12 (52.2%)	1.00	0.007 <sup>a</sup>	59.7	65.2	
		C/T	0 (0%)	2 (8.7%)	NA (0.00–NA)				
		T/T	2 (8.7%)	9 (39.1%)	7.9 (1.5–42.6)				
	Dominant	C/C	21 (91.3%)	12 (52.2%)	1.00	0.002 <sup>a</sup>	58.4	62.1	
		C/T-T/T	2 (8.7%)	11 (47.8%)	9.6 (1.8–50.9)				
	Recessive	C/C-C/T	21 (91.3%)	14 (60.9%)	1.00	0.01 <sup>a</sup>	61.5	65.2	
		T/T	2 (8.7%)	9 (39.1%)	6.8 (1.3–36.0)				
	Overdominant	C/C-T/T	23 (100%)	21 (91.3%)	1.00	0.09	64.9	68.6	
		C/T	0 (0%)	2 (8.7%)	NA (0.00–NA)				
	R (F(x)association)	Log-additive	—	—	—	3.1 (1.3–7.4)	0.004 <sup>a</sup>	59.5	63.2
		Codominant	C/C	21 (91.3%)	12 (52.2%)	1.00	0.009 <sup>a</sup>	59.7	
			C/T	0 (0%)	2 (8.7%)	0.00			
T/T			2 (8.7%)	9 (39.1%)	7.9 (1.5–42.6)				
Dominant		C/C	21 (91.3%)	12 (52.2%)	1.00	0.002 <sup>a</sup>	58.4		
		C/T-T/T	2 (8.7%)	11 (47.8%)	9.6 (1.8–50.9)				
Recessive		C/C-C/T	21 (91.3%)	14 (60.9%)	1.00	0.01 <sup>a</sup>	61.5		
		T/T	2 (8.7%)	9 (39.1%)	6.8 (1.3–36.0)				
Overdominant		C/C-T/T	23 (100%)	21 (91.3%)	1.00	0.4889	64.9		
		C/T	0 (0%)	2 (8.7%)	0.00				
Log-additive		—	—	—	—	3.1 (1.3–7.4)	0.009 <sup>a</sup>	59.5	
McNemar's test		Chi-square equals 5.8 with 1 df					0.02 <sup>a</sup>		

AIC, Akaike information criterion; AMR, antibody-mediated rejection; BIC, Bayesian information criterion; CI, confidence interval; OR, odds ratio.

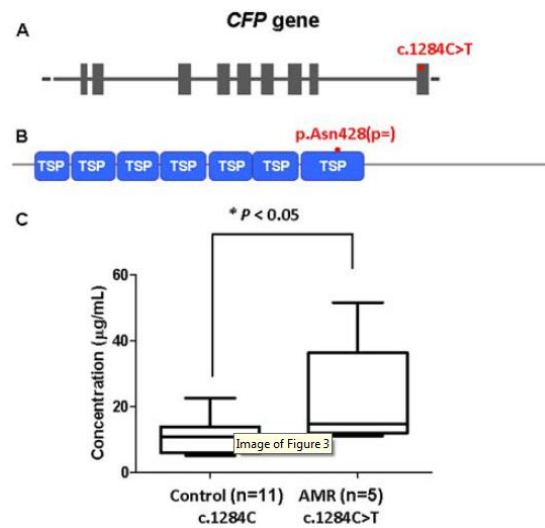
<sup>a</sup>Statistical significance.



The polymorphism in *MBL2* gene is a guanine-to-adenine substitution on the c.161 nucleotide that produces the substitution of the 54 glycine for aspartic acid (p.Gly54Asp) (Figure 2A, B). The MAF (allele = A) described in dbSNP is 0.1389; however, in our patients the rare allele was significantly more frequent in the control patients than in the patients with AMR (Table 2). Although p.Gly54Asp is a polymorphism, 3 software predictions showed that this variant can present an impact in the function of the protein (Figure 2C). Moreover, in the ClinVar database, this allele is described as a pathogenic allele. The second SNP associated with AMR was presented in the *CFP* gene, which codes for properdin protein. It is a cytosine-to-thymine substitution on the c.1284 nucleotide that does not modify the 428 asparagine aminoacid (p. Asn428(p=)) (Figure 3A, B). The MAF (allele = T) described in dbSNP is 0.2287; however, in our patients the rare allele was significantly more frequent in patients who developed AMR than in control patients (Table 3).



**Figure 2.** *MBL2* transcript, MBL primary domains, and MBL protein structure. (A) Schematic structure of the human *MBL2* gene showing relative position of the common polymorphism rs1800450 (c.161G4A). (B) Primary protein domains of MBL and the relative location of the common polymorphism rs1800450 (p.Gly54Asp). The N-terminal cysteine-rich domain (21–41) is shown in orange, the collagen-like domain (42–99) is shown in gray, the coiled coil structure (112–130) is shown in green, and the cytoplasmic tail (134–245) is shown in blue. Red spots (A,B) indicate the polymorphism position. (C) In silico predictions of the effect of p.Gly54Asp variant. (D) MBL serum levels according to *MBL2* genotype among 4 control patients who carried the variant p.Gly54Asp and 5 patients with AMR in which the analyzed SNP was not present.



**Figure 3.** *CFP* transcript and properdin primary domains. (A) Schematic structure of the human *CFP* gene showing relative position of the common polymorphism rs1048118 (c.1284C4T). (B) Primary protein domains of properdin and the relative location of the common polymorphism rs1048118 (p.Asn428(p=)). Seven thrombospondin type-1 (TSP1) domains are shown in blue. Red spots (A,B) indicate the polymorphism position. (C) Properdin serum levels according to *CFP* genotype among 5 patients with AMR who carried the variant and 11 control patients with out the studied variant.

### ***Serum MBL levels in control patients vs patients with AMR correlated with described genotype***

The median MBL serum level measured before HT in 4 patients who did not develop AMR and carried the variant p.Gly54Asp was 6.2 ng/ml (IQR 5.1–6.9 ng/ml) (Figure 2D). This value was significantly lower compared with the median MBL serum level measured before transplantation in 5 patients with AMR in whom the analyzed SNP was not present (207.5 ng/ml (IQR 84.8–290.9 ng/ml),  $p < 0.01$ ). Thus, these findings confirm that control patients who carried the SNP rs1800450 present low MBL levels.

### ***Serum properdin levels in patients with AMR correlated with described genotype***

Median properdin serum levels of 5 patients with AMR who carried the variant rs1048118 was 14.7  $\mu\text{g/ml}$  (IQR 12.0–36.4  $\mu\text{g/ml}$ ), whereas median serum levels of 11 control patients without the studied variant was 10.8  $\mu\text{g/ml}$  (IQR 5.9–13.9  $\mu\text{g/ml}$ ). Thus, the genotype rs1048118 in patients with AMR was positively and significantly associated with higher concentrations of properdin ( $p < 0.05$ ) (Figure 3C).

### ***Secondary association between p.Gly54Asp-MBL2 and p.Asn428(p=)-CFP and CAV***

Owing to the relationship between CAV and AMR,<sup>3</sup> a secondary genetic association study was also carried out to test for an association between CAV and p.Gly54Asp-MBL2 and p.Asn428(p=)-CFP genes, which are associated with AMR. CAV in transplant recipients were classified according to ISHLT categories (CAV1, CAV2, and CAV3).<sup>2</sup> Using the pathologic CAV classification,<sup>26</sup> 8 patients were classified as CAV1, 2 patients were classified as CAV2, and 2 patients were classified as CAV3. The correlation analysis, performed with SNPstats and R software, showed a nonsignificant association for SNP (MBL2 [ $p > 0.99$ ] and CFP [ $p > 0.2$ ]) and CAV.

## **Discussion**

To our knowledge, the present study represents the largest and the most exhaustive study to date on the effect of variants on genes involved in the complement pathway related to AMR in HT recipients. We have identified 2 SNPs, p.Gly54Asp-MBL2, which is associated with absence of AMR, and p.Asn428(p=)-CFP, which is associated with the development of AMR. Moreover, the presence of the rare allele p.Gly54Asp-MBL2 in control patients correlates with an immunodeficiency of MBL, whereas the presence of the rare allele p.Asn428(p=)-CFP in patients with AMR correlates with higher levels of properdin protein.

### ***Protective role of p.Gly54Asp in MBL2 gene from AMR***

p.Gly54Asp is the first SNP significantly and negatively associated with AMR. The higher frequency of this variant in control patients correlates to lower concentration of MBL, which could be classified as MBL deficiency ( $< 100$  ng/ml). MBL2 gene codes for MBL protein, which is the initiator of the complement lectin pathway and a key component of the activation of the innate immune system.<sup>27</sup> The polymorphism p.Gly54Asp presents a MAF between 0.1220 (dbSNP—1000 Genomes) and 0.1389 (ExAC), and the in silico tools used in this study predict a deleterious effect. It has been previously described that the presence of this polymorphism in heterozygous individuals produces an MBL deficiency.<sup>28</sup> This effect is confirmed with our data showing low concentration (median = 6.2 ng/ml) of MBL in our control patients who carry the variant. However, most of the previous studies associated the presence of p.Gly54Asp with decreased host defense against various infectious agents.<sup>27</sup> In the field of transplantation, most studies related to p.Gly54Asp have focused on renal transplant outcomes. The results were inconclusive because some studies failed to find an association between genetic profile of the

lectin pathway of complement activation and allograft outcome in renal transplants,<sup>19,21</sup> whereas others showed a relationship with the incidence of acute rejection and graft outcome.<sup>29,30</sup>

In HT, 2 studies described MBL deficiency related to development of CAV<sup>31</sup> and acute graft rejection.<sup>32</sup> However, neither study analyzed the presence or absence of the variant p.Gly54Asp in the patients. In this regard, another study highlighted that the relationship between MBL deficiency and disease is complex and depends on different genetic and environmental factors, and that could explain why the data obtained in different studies are sometimes contradictory.<sup>33</sup> The present study correlates the genotype p.Gly54Asp with MBL deficiency and indicates a protective effect from AMR, where the complement pathway plays a key role. However, we did not find significant association between this polymorphism and CAV.

### ***Increased risk of AMR in patients with p.Asn428(p=) in CFP gene***

The p.Asn428(p=) in CFP gene (rs1048118) is the second SNP significantly and positively associated with AMR. This variant in patients with AMR correlates with higher concentration of properdin than in control patients. The CFP gene encodes the properdin protein, also known as factor P. Properdin stabilizes the active enzyme complex of the alternative pathway and is the only known positive regulator of complement activation.<sup>34,35</sup> It has been shown that properdin deficiency results in an increased risk of infection; infection mortality rates of 33%–75% have been reported.<sup>36</sup> It has been hypothesized that higher levels of properdin, such as the levels shown in our AMR cohort, result in a higher capacity to activate the alternative pathway and may therefore represent activation of the alternative pathway in relevant situations, such as cardiovascular events,<sup>37</sup> type 2 diabetes mellitus,<sup>38</sup> and AMR. Human studies on the alternative pathway factor properdin in relation to transplantation are scarce. The most relevant article in this area focused on cellular rejection.<sup>39</sup> The authors showed that ISHLT grade 3A heart biopsy specimens (moderate rejection or 2R) contained significantly more transcripts than grade 0 or 1 biopsy specimens for properdin. Taking all these data together, we can hypothesize that high levels of properdin, owing to the presence of the rs1048118 SNP, could have an important role in the development of AMR or cellular rejection.

### ***Limitations of the study***

Limitations of this study include its retrospective single-center design, different criteria of AMR definition, and clinical differences between groups. Moreover, because of the relatively small sample size, we were unable to perform multivariate analysis.

### **Conclusions**

AMR in HT is a complex entity. We have described variants of genes related to the complement system associated with this phenotype. Specifically, the variants *CFP* (p.Asn428 (p=)) and *MBL2* (p.Gly54Asp) are associated with an increase and a reduction of the associated risk of AMR, respectively.

### **Disclosure statement**

None of the authors has a financial relationship with a commercial entity that has an interest in the subject of the presented manuscript or other conflicts of interest to disclose.

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## Supplementary data

Supplementary data associated with this article can be found in the online version at [www.jhltonline.org](http://www.jhltonline.org).

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