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Association of four DNA polymorphisms with acute rejection after kidney transplantation

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Summary

Renal transplant outcomes exhibit large inter-individual variability, possibly on account of genetic variation in immune-response mediators and genes influencing the pharmacodynamics/pharmacokinetics of immunosuppressants. We examined 21 polymorphisms from 10 genes in 237 *de novo* renal transplant recipients participating in an open-label, multicenter study [Cyclosporine Avoidance Eliminates Serious Adverse Renal-toxicity (CAESAR)] investigating renal function and biopsy-proven acute rejection (BPAR) with different cyclosporine A regimens and mycophenolate mofetil. Genes were selected for their immune response and pharmacodynamic/pharmacokinetic relevance and were tested for association with BPAR. Four polymorphisms were significantly associated with BPAR. The *ABCB1* 2677T allele tripled the odds of developing BPAR (OR: 3.16, 95% CI [1.50–6.67]; $P = 0.003$), as did the presence of at least one *IMPDH2* 3757C allele (OR: 3.39, 95% CI [1.42–8.09]; $P = 0.006$). BPAR was almost fivefold more likely in patients homozygous for *IL-10* -592A (OR: 4.71, 95% CI [1.52–14.55]; $P = 0.007$) and twice as likely in patients with at least one A allele of *TNF*-α G-308A (OR: 2.18, 95% CI [1.08–4.41]; $P = 0.029$). There were no statistically significant interactions between polymorphisms, or the different treatment regimens. Variation in genes of immune response and pharmacodynamic/pharmacokinetic relevance may be important in understanding acute rejection after renal transplant.

Introduction

Transplant outcomes exhibit substantial inter-individual variability among patients receiving the same immunosuppressive regimen. Studies have shown that genetic differences may account for some of the variations in renal allograft survival, for example through heat shock protein 70 s and toll-like receptor polymorphisms, [1] as well as variations in patient survival, for example through nucleotide oligomerization domain-2/caspase-recruiting activating domain-15 (NOD2/CARD15) haplotypes [2].

Organ rejection after transplantation may also be subject to genetic variation, for example, in genes involved in the immune response or in the pharmacokinetics/pharmacodynamics of immunosuppressive drugs. The pharmacokinetics of calcineurin inhibitors (CNIs) are influenced by the cytochrome P450 3A enzyme and the multi-drug resistance 1 transmembrane pump (*ABCB1*),

rendering CNI-treated transplantation among the most intensively studied models in pharmacogenetics [3]. Similarly, because of the possible role of cytokines and other immune mediators in transplant rejection, polymorphisms and gene expression profiles of these genes have been widely studied [4–6]. Glander *et al.* [7] reported a correlation between biopsy-proven acute rejection (BPAR) and inter-individual variability of inosine monophosphate dehydrogenase (IMPDH) enzyme activity prior to immunosuppressive treatment, which may be on account of variation in the *IMPDH* gene.

Many effective immunosuppressive agents are available for preventing organ transplant rejection, but most drugs are associated with significant toxicity. CNIs, such as cyclosporine A, successfully reduce the incidence of acute rejection of renal allografts, but their use is associated with nephrotoxicity, limiting their long-term benefit [8]. Therefore, there has been a clinical focus on reducing the dose of or replacement of CNIs, while maintaining an acceptably low rejection rate. The Cyclosporine Avoidance Eliminates Serious Adverse Renal-toxicity (CAESAR) study compared either low-dose cyclosporine or withdrawal of cyclosporine, in conjunction with mycophenolate mofetil (MMF), with a regimen of standard-dose cyclosporine plus MMF in primary renal allograft recipients [9].

In this study, we present an exploratory investigation of the different genetic components that may contribute directly or indirectly to BPAR in a subpopulation of patients from various centers participating in the CAESAR study. We analyzed 21 single nucleotide polymorphisms (SNPs) in 10 candidate genes for association with first BPAR (grade 1 or worse, based on the Banff 93–95 criteria) within the first 12 months post-transplant. The genes selected for investigation are involved in immune response or in the pharmacokinetics/pharmacodynamics of the immunosuppressive agents used in this study.

Materials and methods

Study design and patients

The CAESAR study was a 12 months, prospective, international, multicenter, open-label, randomized controlled trial, which included primary renal allograft patients of low-to-moderate immunological risk. The full inclusion and exclusion criteria, randomization procedure and further details of the study are described elsewhere [9].

Briefly, patients from 32 centers worldwide were randomized in a 1:1:1 ratio to one of three treatment groups:

1 Cyclosporine withdrawal: received daclizumab induction, MMF, corticosteroids, and low-dose cyclosporine (target trough level 50–100 ng/ml until month 3, followed by tapering of dose until complete withdrawal at month 6).

2 Low-dose cyclosporine: received daclizumab induction, MMF, corticosteroids and low-dose cyclosporine (target trough level 50–100 ng/ml for 12 months).

3 Standard-dose cyclosporine: received MMF, corticosteroids and standard-dose cyclosporine (target trough level 150–300 ng/ml until month 4 and 100–200 ng/ml thereafter).

All patients received MMF 2 g/day. Cyclosporine was Neoral or a bioequivalent. The primary end point was measured glomerular filtration rate (GFR) at 12 months post-transplantation; secondary end points included the proportion of patients with first BPAR.

The flow of patients through the CAESAR study has been previously outlined [9]. Of the 535 patients in the intent-to-treat population, informed consent to participate in the pharmacogenetic substudy was given by 275 patients and 237 patients of Caucasian origin (self-reported) were included in the analysis. Patients from other ethnic backgrounds were excluded to reduce possible ancestry-based bias in the analysis.

Data from patients consenting to this genetic substudy were extracted from the database, and then underwent a procedure to make the participants' data anonymous, to ensure that they could not be linked to an individual patient. No individual patient data have been or will be identified or distributed to investigators, patients, or within Roche.

The population characteristics of this substudy are shown in Table 1.

Genes and polymorphisms

Twenty-one SNPs in 10 candidate genes were chosen because: (i) they had previously been associated with the pharmacokinetics of cyclosporine (*CYP3A4, CYP3A5, ABCB1* and *MRP2*); (ii) they are the targets of MMF (*IMPDH1* and *IMPDH2*); or (iii) they are known to be involved in immune response (*IL-2, IL-10, TGF-*β1 and *TNF-*α). The polymorphisms were chosen based on their extrapolated population frequency (see Sequencing and genotyping section). The characteristics of each polymorphism, their incidence in Caucasian nonimmunosuppressed control populations, and rationale for choosing them are outlined in Table 2.

Sequencing and genotyping

Blood samples (9 ml) were collected in EDTA-containing tubes. DNA was extracted from 200 μl of whole blood using a silica adsorption-based extraction method (MagNA Pure LC DNA Isolation Kit I; Roche Applied Science, Indianapolis, IN, USA).

Table 1. Characteristics of the study population.

Baseline characteristics	CsA withdrawal (*n* = 77)	Low-dose CsA (*n* = 86)	Standard-dose CsA (*n* = 74)	Total (*n* = 237)
Gender: males/females, *n* (%)	47/30 (61/39)	55/31 (64/36)	47/27 (64/36)	149/88 (63/37)
Age: ≤50/>50 years, *n* (%)	42/35 (54/46)	47/39 (55/45)	33/41 (45/55)	122/115 (51/49)
HLA-A mismatches 0/1/2, *n* (%)	13/38/26 (17/49/34)	24/41/21 (28/48/24)	19/36/19 (26/49/26)	56/115/66 (24/49/28)
HLA-B mismatches 0/1/2, *n* (%)	7/43/27 (9/56/35)	7/41/38 (8/48/44)	11/34/29 (15/46/39)	25/118/94 (11/50/40)
HLA-DR mismatches 0/1/2, *n* (%)	22/42/13 (29/55/17)	21/46/19 (24/53/22)	25/36/13 (34/49/18)	68/124/45 (29/52/19)
Donor type deceased/living related/living unrelated, *n* (%)	63/7/7 (82/9/9)	76/2/8 (88/2/9)	68/2/4 (92/3/5)	207/11/19 (87/5/8)
Donor age ≤55/>55 years, *n* (%)	58/19 (75/25)	67/19 (78/22)	59/15 (80/20)	184/53(78/22)
Cold ischemic time 0–30 h/>30 h, *n* (%)	76/1 (99/1)	84/1 (99/1)	72/2 (97/3)	232/4 (98/2)
Patients with most recent PRA >20%, *n* (%)*	0	1 (1.2)	0	1 (1.2)
First BPAR				
BPAR at 3 months yes/no/missing data† , *n* (%)‡	7/69/1 (9)	17/69/0 (20)	17/56/1 (23)	41/194/2 (17)
BPAR at 12 months yes/no/missing data†, *n* (%)‡	22/51/4 (30)	25/61/0 (29)	17/56/1 (23)	64/168/5 (28)
GFR at 12 months: mean [95% CI] (ml/min/1.73 m^2)	53.13 [48.17–58.09] (64)	53.31 [48.70–57.93] (70)	54.58 [50.89–58.81] (53)	53.69 [51.06–56.31] (187)

BPAR, biopsy-proven acute rejection incidence (defined according to Banff 93–95 criteria excluding borderline changes); CsA, cyclosporine A; GFR, glomerular filtration rate; PRA, panel reactive antibodies.

*Missing data for two patients in low-dose CsA and one patient in standard-dose CsA.

†Missing data on patients who dropped out of the study prior to month 12 and had no acute rejection episode during the observation period.

‡Percentage of BPAR incidence per treatment group is calculated excluding individuals with missing information.

Exons and parts of the 5′- and 3′-regions of the genes were sequenced on a panel of 47 DNAs obtained from the Coriell Institute (HD4 panel) to screen for common SNPs not reported in the literature (data not shown).

Samples were genotyped for 14 SNPs (Table 2) using allele-specific polymerase chain reaction (PCR) using an ABI GeneAmp 7900 Sequence Detection System instrument [27]. Genotyping for seven SNPs (Table 2) was done by double-stranded DNA sequencing using an ABI capillary sequencing apparatus and 'Big Dye' chemistry (ABI). Cycle sequencing was performed on an MJ Tetrad PCR machine using ABI Big Dye terminator chemistry according to the manufacturer's instruction. After sequencing, the polymorphism analyses were performed using Polyphred software (licensed from University of Washington).

Primer sequences and amplification conditions may be requested from Dr O. Spleiss.

Statistical methods

Following a review of all available baseline characteristics (Table 1) potentially impacting BPAR, two logistic models were constructed with the same set of explanatory variables, taking rejection events at 3 and 12 months as the response variable. The following potential risk factors of rejection were selected as they were found to be associated with BPAR in either of the two models, using a loose significance level ($P = 0.1$): treatment group, gender, recipient age [≤50/>50 years] and donor type.

The genotypic association between each of the 21 polymorphisms and BPAR at 1 year was examined using a logistic regression model adjusting for the baseline risk factors listed above (Table 3). The absence of association between the genotypes and BPAR was tested using a Wald test. When the frequency of homozygosity for the minor allele was <5%, data of the minor allele homozygous category was pooled with the heterozygous category. Accordingly, the Wald test followed a chi-square with one or two degrees of freedom. The type I error of each genotype test was set to 0.05. No multiple testing adjustments were made, given the exploratory nature of the study.

When genotypic effect significance was detected, additional tests were performed for dominant, recessive and allelic effects. The best-fitting model was used to compute the odds ratio (OR) and 95% confidence interval (CI). Power calculations were conducted as described previously [28]. Additionally, the interaction between the polymorphism and treatment group on BPAR was tested. When more than one polymorphism from the same gene

Table 2. Genes and polymorphisms interrogated.

Gene name	SNP name (MAF)	NIH identifier (MAF)	Rationale
Cytochrome P450 3A4 (*CYP3A4*)	**A**-392G (*1B)[a] (0.95)	rs4986914 (0.98)[d]	Enzyme involved in the metabolism of cyclosporine A (CsA); *1B is the most common and functionally important variant among Caucasians [16].
Cytochrome P450 3A5 (*CYP3A5*)	A6986**G** (*3)[a] (0.91)	rs776746 (0.94)[e]	Enzyme involved in the metabolism of CsA; *3 is the most common and functionally important variant in Caucasians [16].
Multi-drug resistance gene type I (*ABCB1/MDR1*)	**C**3435T[a] (0.51) **A**417G (exon 28)[a,b] (0.87) **A**61G[c] (N21D) (0.91) **C**1236T[a] (0.6) **G**2677T/A[c] (0.58)	rs1045642 (0.54)[e] rs3842 (0.86)[g] rs9282564 (0.91)[e] rs1128503 (0.61)[e] rs2032582 (0.46)[e]	CsA is a substrate of P-glycoprotein encoded by *ABCB1*. Higher CsA clearance has been found in renal transplant patients carrying the 3435T allele [17]. *ABCB1* C3435T has been associated with acute persistent rejection in the first post-operative year in lung transplant patients [18].
Multi-drug resistance associated protein 2/adenosine triphosphate-binding cassette, subfamily c, member 2 (*MRP2/ABCC2*)	T3972**C**[c] Exon 28 (0.64) **A**5′ reg6170G[a] (0.58) **T**3600A[b,c] (0.93)	rs3740066 (0.66)[e] rs2804402 NA rs17222723 (0.93)[e]	*MRP2* is involved in the biliary excretion of mycophenolic acid glucuronide. CsA interferes with the biliary clearance by inhibiting the multidrug resistance-associated protein 2 [19].
Inosine monophosphate dehydrogenase type I (*IMPDH1*)	**G**1320A[a,b] (0.78)	rs2228075 NA	MPA is a selective inhibitor of inosine monophosphate dehydrogenase (IMPDH).
Inosine monophosphate dehydrogenase type II (*IMPDH2*)	**T**3757C[b,c] (0.93)	rs11706052 (0.9)[e]	MPA is a selective inhibitor of IMPDH. Among patients with treatment-related toxicities a trend was shown towards higher level of IMPDH2 expression compared to those without toxicity [7]. Low IMPDH activity has been associated with rejection in renal transplants treated with MMF [20].
Interleukin 2 *(IL-2*)	G-330**T**[a] (0.68) **G**114T[a] (0.70)	rs2069762 (0.69)[f] rs2069763 (0.76)[f]	Association of the gene with acute rejection and graft vascular disease after clinical heart transplantation [21]. IL2/-330T has been shown to influence the production levels of the cytokine [22].
Interleukin 10 (*IL-10*)	G-1082**A**[a] (0.55) **C**-592A[a] (0.75) **C**-819T[c] (0.77) **C**-851T[a] (0.98)	rs1800896 (0.62)[5] rs1800872 (0.74)[5] rs1800871 (0.74)[5] rs1800894 (0.95)[e]	*IL-10* promoter genotype G -1082A has been associated with acute rejection episodes in kidney and heart transplant recipients [18]. Among recipients of hematopoietic cells from an HLA-identical sibling, the IL10 C-592A allele has been associated with graft versus host disease [23].
Transforming growth factor-beta 1 (*TGF*-β1)	C915**G** (R25P)[a] (0.92) C869**T** (P10L)[c] (0.61)	rs1800471 (0.89)[f] rs1982073 (0.62)[h]	High responder (Arg25:GG) may correlate with the induction of tolerance following renal transplantation [24]. Association between the *TGF*-β1 genotype encoding proline at codon 10 and renal dysfunction after clinical heart transplantation [25].

Table 2. continued

Gene name	SNP name (MAF)	NIH identifier (MAF)	Rationale
Tumor necrosis factor-alpha (TNF-α)	**G**-308A[a] (0.87)	rs1800629 (0.83)[f]	The variability of immune response may be related to cytokine production. G-308A allele has been associated with up to sevenfold increase in (TNF-α) [26] and a fivefold increase in risk of rejection [27].

CsA, cyclosporine A; MAF, major allele frequency; the major allele is in bold; SNP, single nucleotide polymorphisms. NA, not available; MPA, mycophenolic acid.
[a]Genotyping by allele specific PCR [10].
[b]Polymorphism in gene without prior evidence of association to immune response or transplantation outcome; included after sequence detection.
[c]Genotyping by double-stranded DNA sequencing.
[d]MAF calculated from 79 Caucasian samples [11].
[e]MAF calculated in the Cold Spring harbor Lab Hapmap CEU panel [12].
[f]MAF calculated in the NCI SNP500Cancer CAUC1 panel [13].
[g]MAF calculated in the Coriell Cell repository European panel [14].
[h]MAF calculated from the Coriell Utah Pedigree E-0 [15].

was associated with BPAR, the evaluation of their corresponding effect was performed using a stepwise logistic regression procedure, using backward selection [29]. Again, no adjustments were made for multiple testing.

For genes where multiple SNPs were genotyped, haplotype-based analyses were also performed. Haplotype frequency estimation was performed using the Haplo.stats package [30] and haplotype effect testing was performed using the logistic regression framework developed by Lake *et al.* [31].

Linkage disequilibrium (as measured by r^2) was computed for each pair of SNPs within each gene [32]. Each polymorphism distribution was following the Hardy–Weinberg equilibrium [32] and could thus be included in the haplotype-based analysis. All computations were performed using R 2.2.1 [33].

Results

In the substudy population, there were 77, 86 and 74 patients in the cyclosporine withdrawal, low-dose cyclosporine, and standard-dose cyclosporine groups, respectively. Although the CAESAR study population was not restricted by ethnicity, only Caucasian patients were included in the substudy. There were no notable differences in baseline demographic characteristics, with the exception of the proportion of deceased donors between patients included in this substudy (Table 1) and the overall CAESAR study patient population [9]. There were no major differences in the overall incidence of first BPAR and in renal function (as measured by GFR) at 12 months after transplantation between the substudy population (Table 1) and the overall CAESAR study population [9].

In addition to the absence of a significant deviation from the Hardy–Weinberg equilibrium (data not shown), the similarity of allelic frequencies from our sample to Caucasian nonimmunosuppressed random population controls, as shown in Table 2, suggests that the reported ethnicity in our sample seems accurate and that a risk of ethnic stratification bias in our sample is unlikely.

Four polymorphisms, *ABCB1* G2677T/A, *IMPDH2* T3757C, *IL-10* C-592A and *TNF-α* G-308A, demonstrated a statistically significant association with BPAR at 12 months post-transplant (Table 3).

There were no statistically significant associations between any of the polymorphisms tested and treatment group (with BPAR at 3 and 12 months as the response variable; Table 4 [data pertaining to month 12 only presented]).

ABCB1

In the screening phase, three polymorphisms in *ABCB1* (C3435T, C1236T and G2677T/A) were significantly associated with BPAR (Table 3). These three polymorphisms are in high linkage disequilibrium, especially C1236T and G2677T/A ($r^2 = 0.82$ when the G and A allele of G2677T/A are pooled). A backward selection approach, evaluating the effects of the genetic covariates only, was adopted to distinguish between functional and surrogate markers. We used a logistic regression model combining the baseline risk factors that showed loose association with BPAR (treatment group, gender, recipient age, and donor type) and the three polymorphisms, coded in their best-fit model. When adjusted on the effect of G2677T/A, the main effects of C3435T and C1236T were not significant ($P = 0.41$) demonstrating that G2677T/A was driving the association with BPAR, with C3435T and C1236T likely to be surrogate markers. These three *ABCB1* polymorphisms (C3435T, C1236T and G2677T/A) were also sig-

Table 3. Association between candidate gene polymorphisms and first biopsy-proven acute rejection (BPAR) at 12 months post-transplant adjusted for baseline risk factors*.

Gene position and genotypes	BPAR† at 12 months: No. patients	BPAR incidence (%)	Odds ratio	*P*-value*
CYP3A4				
A-392G				
GG or AG	20	25.0	0.960	0.942
AA‡	195	28.7	–	
CYP3A5				
A6986G				
AA or AG	37	24.3	0.790	0.587
GG‡	186	28.5	–	
ABCB1				
A61G (N21D)				
GG or AG	37	32.4	1.46	0.362
AA‡	180	26.7	–	
C3435T				
TT	53	37.7	4.19	0.0099
CT	117	29.9	2.6	
CC‡	56	16.1	–	
A417G				
GG or AG	53	18.9	0.519	0.099
AA‡	167	29.9	–	
C1236T				
TT	41	43.9	3.9	0.009
CT	103	27.2	1.64	
CC	87	20.7	–	
G2677T/A				
TT	39	43.6	4.93	0.003
GT	103	29.1	2.37	
GA or TA	10	10	0.446	
GG	73	17.8	–	
MRP2				
G5′reg 6170A				
GG	40	27.5	1.26	0.853
AG	104	28.8	1.18	
AA‡	76	26.3	–	
T3600A				
AA or AT	30	16.7	0.363	0.053
TT‡	189	29.1	–	
T3972C				
TT	31	25.8	1.38	0.63
CT	96	30.2	1.37	
CC‡	94	24.5	–	
IMPDH1				
G1320A				
AA	15	33.3	1.0	0.571
AG	67	23.9	0.687	
GG‡	134	29.1	–	
IMPDH2				
T3757C				
CC or CT	28	46.4	3.39	0.006
TT‡	193	24.9	–	
IL-2				
T-330G				
GG	24	25	0.892	0.919
GT	94	28.7	1.06	
TT‡	105	27.6	–	
G114T				
TT	18	44.4	3.1	0.107
GT	97	26.8	0.885	
GG‡	109	25.7	–	
IL-10				
C-592A				
AA	18	55.6	4.55	0.024
AC	72	25	0.897	
CC‡	127	25.2	–	
A-1082G				
GG	46	28.3	0.940	0.550
AG	105	23.8	0.688	
AA‡	69	33.3	–	
C-819T				
TT	13	53.8	4.23	0.104
CT	68	26.5	1.06	
CC	119	24.4	–	
C851T				
CT or TT	6	16.7	0.460	0.465
CC	190	26.8	–	
TGF-β1				
C869T (P10L)				
CC	37	29.7	1.40	0.214
CT	103	33	1.86	
TT‡	84	21.4	–	
C915G (R25P)				
CC or CG	37	32.4	1.2	0.662
GG‡	187	27.8	–	
TNF-α				
G308A				
GA or AA	52	38.5	2.18	0.030
GG	170	24.7	–	

Table 3. continued

*P-value for the association test derived from the logistic regression using recipient age, gender, treatment group and donor type as covariates (see Materials and methods).
†BPAR grade 1 or worse according to Banff 93–95 criteria.
‡Genotype used as reference category for calculating odds ratios.

nificantly associated with BPAR at 3 months post-transplant (data not shown).

Patients homozygous for the *ABCB1* 2677T allele were more than twice as likely to experience BPAR as GG-homozygous patients. The best-fitting genetic model was a multiplicative allelic model and using this, adjusted for the baseline risk factors, the T allele was associated with a

threefold increase of experiencing BPAR (OR: 3.16, 95% CI [1.50–6.67]; $P = 0.003$) compared with the G or A alleles (Table 4).

In addition, haplotype-based analysis [31] combining the three *ABCB1* SNPs was performed and the effect of each individual haplotype was tested, adjusted on the baseline risk factors. The haplotype T-T-T occurred at a frequency of 41.4% in patients with BPAR compared with 24.4% among patients with no rejection episode, and significantly increased the odds of rejection relative to the C-G-C reference haplotype (OR: 2.14; [1.34–3.44] $P = 0.002$).

IMPDH2

As there were only two CC carriers (1%) of the *IMPDH2* T3757C polymorphism, the CC and CT genotypes were pooled and only one genetic coding scheme was investigated. Patients carrying one or two C alleles were three times more likely to experience BPAR at 3 months than TT homozygous carriers (data not shown). This increased slightly at 12 months (OR: 3.39, 95% CI [1.42–8.09]; $P = 0.006$; Tables 3 and 4).

IL-10

Only the C-592A polymorphism in the *IL-10* gene showed significant association to BPAR (Table 3). The BPAR rate was more than fourfold higher in AA homozygous patients than for CC homozygous patients (Table 3). The recessive model was the best-fitting model, demonstrating an OR for BPAR in AA-homozygous patients of 4.71 (95% CI [1.52–14.55]; $P = 0.007$) compared with AC/CC-genotype carriers (Table 4).

The joint haplotypic analysis of all three *IL-10* SNPs showed that the A-T-A haplotype occurs in 27% of patients with BPAR compared with 16.7% of patients without BPAR. In a logistic regression analysis using a multiplicative model, the effect of this haplotype was close to significance when tested against the reference haplotype G-C-C ($P = 0.07$). Using a recessive model, the effect of the A-T-A haplotype was significant ($P = 0.03$). Given the very tight linkage disequilibrium between C-592A and C-819T, it is almost impossible to differentiate the effects of these markers. In addition, as the -592A allele is present in only one common haplotype (A-T-A), the specific effect of the allele and of this haplotype is impossible to differentiate. Using our logistic model, we could rule out the effect of G-1082A adjusted on the effect of C-592A or C-819T (coded in a recessive model). There was no association of *IL-10* with BPAR at 3 months.

Table 4. Logistic regression results on biopsy-proven acute rejection for each of the statistically significant single nucleotide polymorphisms at 12 months including baseline risk factors†.

Effect	OR [95% Wald CI]	*P*-value
***ABCB1* G2677T**		
Age (≤50/>50 years)	2.067 [1.078–3.963]	0.0289
Sex (female/male)	0.446 [0.223–0.892]	0.0224
Treatment‡		
CsA withdrawal versus standard CsA	1.331 [0.589–3.007]	0.4912
Low versus standard CsA	1.292 [0.595–2.803]	0.5169
Donor type: live related	0.565 [0.111–2.876]	0.4913
Donor type: live unrelated	4.095 [1.408–11.910]	0.0097
G2677T/A (T versus G/A)	3.159 [1.497–6.668]	0.0025
G2677*treatment‡	–	0.8375
***IMPDH2* T3757C**		
Age (≤50/>50 years)	2.389 [1.245–4.590]	0.0089
Sex (female/male)	0.478 [0.237–0.94]	0.0325
Treatment‡		
CsA withdrawal versus standard CsA	1.507 [0.665–3.42]	0.3258
Low versus standard CsA	1.309 [0.602–2.85]	0.4963
Donor type: live related	0.483 [0.093–2.52]	0.3886
Donor type: live unrelated	2.908 [1.032–8.19]	0.0434
T3757C (CC,CT/TT)	3.39 [1.417–8.09]	0.0061
T3757C*treatment	–	0.1694
***IL-10* C-592A**		
Age (≤50/>50 years)	2.298 [1.188–4.446]	0.0135
Sex (female/male)	0.362 [0.170–0.771]	0.0085
Treatment		
CsA withdrawal versus standard CsA	1.172 [0.516–2.665]	0.7044
Low versus standard CsA	1.073 [0.491–2.343]	0.8596
Donor type: live related	0.499 [0.094–2.652]	0.4143
Donor type: live unrelated	2.755 [0.961–7.901]	0.0594
C-592A [AA/(AC,CC)]	4.706 [1.522–14.554]	0.0072
C-592A*treatment	–	0.2668
***TNF-α* G-308A**		
Age (≤50/>50 years)	2.333 [1.230–4.428]	0.0095
Sex (female/male)	0.493 [0.252–0.966]	0.0394
Treatment‡		
CsA withdrawal versus standard CsA	1.439 [0.651–3.181]	0.3681
Low versus standard CsA	1.048 [0.485–2.264]	0.9054
Donor type: live related	0.386 [0.073–2.041]	0.2627
Donor type: live unrelated	2.885 [1.057–7.877]	0.0387
G308A [(AA,AG)/GG]	2.184 [1.083–4.406]	0.0291
G308A*treatment	–	0.1959

CI, confidence interval; CsA, cyclosporine A; OR, odds ratio.

*Test for statistical significance of polymorphism-by-treatment interaction.

†Baseline characteristics identified as potential risk factors for BPAR with marginal significance ($P = 0.1$) at initial modeling: recipient age, gender, treatment group and donor type.

‡Treatment group definition: see Materials and methods section. Standard CsA group was used as the reference group.

TNF-α

The frequency of BPAR was twofold higher in patients with at least one A allele of *TNF-α* G-308A (OR: 2.18, 95% CI [1.08–4.41]; $P = 0.029$; Tables 3 and 4). As there were only six carriers of the AA genotype (2%), AA and AG were pooled and only one genetic coding scheme was investigated. Of these six homozygous subjects, only one experienced BPAR, showing that the significant increased risk of BPAR in the AA/AG group was most likely induced by the AG heterozygous genotype. There was no association of *TNF-α* G-308A with BPAR at 3 months.

Discussion

This analysis studied a subpopulation of patients from various centers participating in the CAESAR study, unlike many pharmacogenetic studies that are based on samples from a single clinical center, and represents one of the largest investigations of the role of genetic variants in solid organ transplantation.

Our strategy was to identify genetic factors of rejections, adjusted on known or suspected demographic or clinical characteristics. Given the number of potential associated factors (Table 1), we preselected those bearing at least some association with the outcome in our dataset (recipient age, treatment group, gender and donor type). Second, we confirmed that polymorphisms were evenly distributed across treatment groups, thereby eliminating the different rejection risks associated with treatment effects. Third, by focusing on patients with the self-reported Caucasian ethnicity, comparing the estimated allelic frequencies in our dataset with allelic frequencies of Caucasians in the general population database and by checking the Hardy–Weinberg equilibria for each of the SNPs, we can reasonably conclude that it is unlikely that ethnic stratification is a confounding factor in this study.

Using this strategy, four polymorphisms were found to be significantly associated with BPAR at 12 months statistically, with two of them also showing an association at 3 months post-transplant.

ABCB1

The *ABCB1* gene codes for P-glycoprotein, which functions to ensure the energy-dependent cellular efflux of substrates. As cyclosporine is also a substrate of P-glycoprotein, a substantial proportion of the variability in absorption and clearance of cyclosporine has been attributed to variability in intestinal P-glycoprotein concentration and activity.

We demonstrated that the *ABCB1* 2677T allele increased the incidence of BPAR threefold and that the *ABCB1* SNPs (C3435T, C1236T and G2677T/A) are in high linkage disequilibrium, consistent with previous studies [34,35]. However, our further analysis concluded that the association was driven by G2677T/A, with C3435T and C1236T being likely surrogate markers. Conflicting data have been previously presented on the most extensively studied SNP in *ABCB1*, C3435T. The T-variant has been reported as both increasing and decreasing P-glycoprotein function [36–38] and its role regarding cyclosporine dose requirements and clearance in renal transplant patients is controversial [13,39–41]. The tight linkage of the three SNPs resulting in the C3435T being a surrogate marker as demonstrated in our study, could offer one possible explanation for these controversies.

The interactions of these three *ABCB1* SNPs are complex, and the importance of haplotype must be considered. Here, we showed that the frequency of the C-G-C haplotype was 38%, and the frequency of the T-T-T haplotype was 29%, similar to frequencies shown in a previous study in a Caucasian population [34], thereby ruling out the potential confounding effects of ancestry-based bias. The T-T-T haplotype significantly increased the odds of BPAR relative to the C-G-C reference haplotype, which is consistent with observations regarding the incidence of BPAR associated with each allele and with the pattern of linkage disequilibrium among the three *ABCB1* polymorphisms. A previous *ABCB1* haplotype analysis suggested that the T-T-T haplotype shows a weak (nonsignificant) trend towards a greater exposure to cyclosporine, and the authors suggested that the *ABCB1* SNPs are unlikely to be useful for cyclosporine dose optimization [42]. In our study, we used a combination of different drugs and it is possible that these complex interactions will require further study.

Despite the relationship between *ABCB1* and cyclosporine pharmacokinetics, in this analysis we did not show a relationship between *ABCB1* polymorphisms and the different treatment groups. This is surprising as each of the treatment groups had different target blood trough levels of cyclosporine. In the case of the cyclosporine withdrawal group, this may be related to the fact that some of the patients experienced acute rejection after cyclosporine withdrawal and subsequently received cyclosporine or tacrolimus [9]; thus the treatment received by patients in this group was quite heterogeneous. However, this was not the case for the low-dose and standard-dose cyclosporine groups who were generally maintained on their allocated treatment regimens.

Using data from a CAESAR pharmacokinetic substudy, we attempted to correlate these pharmacogenetic data with pharmacokinetic parameters. However, only a very small number of individuals had both pharmacogenetic and pharmacokinetic data available, which jeopardized the anonymous nature of the patient data. In addition, pharmacokinetic data were only available at four preset time points, not when rejections occurred, meaning that no conclusions could be drawn.

IMPDH2

The active metabolite of MMF, mycophenolic acid (MPA), is a potent, selective inhibitor of IMPDH and, therefore, inhibits the *de novo* pathway of purine synthesis in T and B lymphocytes [43]. Here, we demonstrated a previously unreported association: a polymorphism in intron 7 of *IMPDH2*, T3757C, was associated with approximately three-times higher odds of experiencing BPAR at 12 months. The possible effects of this polymorphism, located seven bases downstream of the 3′ end of exon 6, are unknown. Our finding appears to support the hypothesis that *IMPDH* variants are relevant for MPA pharmacodynamics, although as previously discussed, we have been unable to directly demonstrate this on account of the small numbers of patients with pharmacogenetic and pharmacokinetic data. However, it was established that patients experiencing BPAR in the CAESAR study were overall receiving lower average daily doses of MMF [9].

Large inter-individual variation of IMPDH enzymatic activity before transplantation has been observed [7], which may be responsible for some of the side-effects associated with MMF therapy. Further studies are needed to determine whether the T3757C polymorphism is correlated with IMPDH enzyme activity. In this context, finding an association of BPAR with a polymorphism in the gene encoding the type II isoform of IMPDH, as opposed to the type I isoform, would plausibly be expected, as MPA is five-times more potent at inhibiting the type II isoform than the type I isoform, and the inducible type II isoform is strongly expressed in activated lymphocytes [20]. Nonetheless, the association of this polymorphism with BPAR may not be fully explained by the inter-individual variability of IMPDH enzyme activity. Recently, using a similar assay as that used by Glander *et al.* [7], van Gelder *et al.* did not find a correlation between *IMPDH2* T3757C and IMPDH enzyme activity (T. van Gelder, personal communication). However, in a transplantation cohort, a borderline-significant association between this polymorphism and BPAR at 1 year has been demonstrated (T. van Gelder, personal communication).

IL-10

Interleukin-10 is an immunomodulatory cytokine, with immunosuppressive and anti-inflammatory effects. Our study is the first to demonstrate an association between the *IL-10* C-592A polymorphism and BPAR in kidney transplantation, possibly pointing to a role of immune response variance in the incidence of BPAR. Patients homozygous for the A allele at the *IL-10*-592 site had a statistically significant fivefold increased risk of BPAR at 12 months post-transplant, independent of baseline risk factors, when compared to those carrying one or two of the C-alleles. Interestingly, there was not a statistically significant association between this polymorphism and BPAR at month 3 post-transplant, indicating that this association is driven by late rejections (data not shown).

In concordance with our observations, a recent study in a similar population [27] found patients homozygous for the -592A allele had a higher risk of developing BPAR (OR: 2.2, 95% CI [1.0–4.7]; $P = 0.072$). Conversely, a report in 209 transplant recipients did not find any association between C-592A polymorphism and BPAR [44] although this study only considered BPAR during the first 30 days post-transplantation under a different treatment regimen to that in the CAESAR study. The AA genotype was recently reported to be associated with a lower incidence of graft versus host disease following bone-marrow transplantation [23]. This supports the hypothesis that AA homozygous carriers may be more likely to mount an immune response upon challenge. In the context of transplantation, such patients may be more at risk of late rejections as their immunosuppressant load is gradually reduced over time.

Several mechanisms of a functional effect of the *IL-10* C-592A polymorphism are plausible. First, it is positioned between two putative consensus binding sequences for the transcription activator, SP1, and its family-like proteins [45]. Further, this polymorphism has been shown during *in vitro* experiments, independent of its haplotype context, to influence *IL-10* transcription levels [46]. This points to a complex interrelationship among the C-592A polymorphism, *IL-10* mRNA and protein synthesis, and their possible association with a reduced immune tolerance [47]. However, as a note of caution regarding this association, in individuals of Caucasian and Asian ancestry, the -592A allele occurs only in the context of one frequent haplotype [23], not permitting a distinction between the specific effect of this polymorphism and the joint effects of the other alleles contributing to this haplotype.

Interleukin-10 promoter SNPs have been extensively investigated in genetic studies in transplantation and auto-immune and infectious diseases, notably HIV-1

progression [48] and their association with *IL-10* mRNA levels or promoter activity has shown conflicting results [5,45,49]. The polymorphism studied most frequently in renal transplantation (again with widely varying outcomes) is the G-1082A polymorphism [50]. We did not find any association between this polymorphism and BPAR in our dataset and could rule out the effect of G-1082A adjusted on the effect of C-592A or C-819T. The cluster formed by these two tightly linked SNPs seems to explain the association of *IL-10* with rejection.

TNF-α

The association of *TNF-α*, a pro-inflammatory cytokine, with graft rejection in solid organ transplants has been previously studied [26,27], and the -308A allele has been associated with rejection in transplant recipients [27]. At 12 months post-transplant, we identified a statistically significant twofold increase in BPAR when the A allele of this polymorphism was present. However, as there was no statistically significant association at 3 months post-transplant, the *TNF-α* G-308A polymorphism appears to be more important in late acute rejections. As with the *IL-10* C-592A polymorphism, this *TNF-α* G-308A polymorphism may be most important as a patient's immunosuppressive load is reduced as time elapses after transplantation.

Our result at 12 months post-transplant seems to confirm that the patients with the *TNF-α* -308 GA or AA genotype are at higher risk of acute rejection. However, it should be noted that we combined data from GA heterozygotes and AA homozygotes on account of the small number of AA homozygotes in our sample. As a result, we cannot fully corroborate the results of Alakulppi *et al.* [27] that described a significant association with AA homozygotes. There have also been conflicting reports about the *TNF-α* G-308 allele and the risk of rejection when linked to the *IL-10*-AA polymorphism [26,27,44].

Clinical applications of associations of polymorphisms with BPAR

What are the practical consequences of the results of the current study with regard to transplant patient management? Can the polymorphic markers identified as associated with BPAR be used for patient stratification according to risk of transplant rejection and, thus, for a more informed approach towards their medical management? As Pepe *et al.* [51] discussed recently, a marker showing association at an OR of as 'high' as 3 is likely insufficient for stratification of individual patients, depending on the indication and intended use (e.g. prediction of efficacy versus adverse events). For example, based on the results of the current study, the *IL-10* C-592A allele shows a high specificity for BPAR (94.9%), but would identify only a low fraction of patients potentially at high risk for BPAR (sensitivity of 16.7%). While a positive test would, therefore, be useful to classify a patient overall as 'high risk', a negative test would not be useful to identify a 'low risk' patient. Given the cautious default approach to transplant patients, a clinically meaningful test, which would provide useful guidance for reducing immunosuppressive medication, should provide the opposite characteristics, namely high sensitivity, even if at the cost of lower specificity. Yet, if one were to embark on a program of measured reduction of immunosuppressive drugs, or the use of new immunosuppressive regimens, a case could be made out to consider polymorphisms that are known to be associated with a higher risk of rejection (e.g. the *IL-10* polymorphism) as exclusion markers to identify patients who should *a priori* not be considered for such an approach because of their higher risk of an unfavorable outcome.

The lack of replication of candidate genes studies often undermines their validity. This lack of replication is mainly attributable to the small sample size often artificially counter-balanced by the absence of correction for the approach used (multiple testing, inappropriate design, etc.). The plausibility of the conclusions drawn from any molecular studies without any associated independent replication are thus central in genetic epidemiology and are tightly linked to prior plausibility of the hypotheses tested. Previous studies [52] have provided a simple framework to weight the plausibility of results obtained from molecular studies based on the odds of prior assumptions, type I and type II errors. In this study, because of the careful choice of candidate genes and polymorphisms tested and the previous results obtained in this field (Table 2), one can reasonably assume that the ratio of true to false hypotheses is in the range of 1%. Additionally, a power calculation that was performed for the four associated SNPs showed that our power ranged between 0.54 and 0.8 at our type I error. As noted by Thomas and Clayton [53], very small type I errors are required if the prior odds of the tested hypotheses are low. In our case, having a type I error of 5% is in the range of our assumed prior odds, and, together with a reasonable power estimation, this ensures that additional information is brought by our results.

However, our results as they stand are not conclusive. Patients enrolled in the CAESAR study were immunologically low-risk patients. The polymorphisms we have identified that are associated with BPAR may not necessarily be found in high-risk patients or in patients receiving different immunosuppressive regimens. In addition, our findings need to be confirmed in studies

that test them as prespecified hypotheses and further study is required to investigate whether combinations of the markers we identified and other predictive factors can adequately identify patients at low or high risk of acute rejection.

Authorship

JG: Contributed to the clinical study, discussing the data from the pharmacogentic subanalysis and the writing of the manuscript. YV: Contributed to the clinical study and discussing the data from the pharmacogentic subanalysis. BN and HE: Contributed to the clinical study, discussing the data from the pharmacogentic subanalysis and writing and discussion of the manuscript. FV: Contributed to the clinical study and discussing the data from the pharmacogentic subaanalysis. LE: Contributed to designing the pharmacogenetic subanalysis, performing the analysis of the data, writing and discussing the paper. MR and KL: Contributed to designing the pharmacogenetic subanalysis and discussing the paper. CN-M and AV: Contributed to designing the pharmacogenetic subanalysis, writing and discussing the paper. OS: Contributed to performing the analysis of the data and discussing the paper. MT: Contributed to designing the pharmacogenetic subanalysis, performing the analysis of the data and discussing the paper.

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