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The Immunobiogram, a novel in vitro diagnostic test to measure the pharmacodynamic response to immunosuppressive therapy in kidney transplant patients

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ABSTRACT

Background: Diagnostic tools to measure the response to individual immunosuppressive drugs for transplant patients are currently lacking. We previously developed the blood-based Immunobiogram bioassay for in-vitro characterization of the pharmacodynamic response of patients' own immune cells to a range of immunosuppressants. We used Immunobiogram to examine the association between patients' sensitivity to their prescribed immunosuppressants and clinical outcome.

Methods: We conducted an international, multicenter, observational study in a kidney transplant population undergoing maintenance immunosuppressive therapy. Patients were selected by clinical course poor [PCC] $N = 53$ (with renal dysfunction, and rejection signs in biopsy or/and an increase in DSA strength in last 12 months) versus good [GCC] $N = 50$ (with stable renal function and treatment, no rejection and no DSA titers). Immunobiogram dose-response curve parameters were compared between both subgroups in patients treated with mycophenolate, tacrolimus, corticosteroids, cyclosporine A or everolimus. Parameters for which significant intergroup differences were observed were further analyzed by univariate and subsequent multivariate logistic regression.

Results: Clinical outcome was associated with following parameters: area over the curve (AOC) and 25% (ID25) and 50% (ID50) inhibitory response in mycophenolate, tacrolimus, and corticosteroid-treated subgroups, respectively. These statistically significant associations persisted in mycophenolate (OR 0.003, CI95% <0.001–0.258; $p = 0.01$) and tacrolimus (OR < 0.0001, CI95% <0.00001–0.202; $p = 0.016$) subgroups after adjusting for concomitant corticosteroid treatment, and in corticosteroid subgroup after adjusting for concomitant mycophenolate or tacrolimus treatment (OR 0.003; CI95% <0.0001–0.499; $p = 0.026$).

Abbreviations: ABMR, antibody-mediated rejection; AOC, area over the curve; AUC, area under the curve; AZA, azathioprine; CI, confidence interval; CSA, cyclosporine A; dnDSA, de novo donor-specific antibodies; ESRD, end-stage renal disease; EVER, everolimus; GCC, good clinical course; GCP, Good Clinical Practice; ID25, 25% maximal inhibitory response; ID50, half-maximal inhibitory response; ID75, 75% maximal inhibitory response; IMBG, Immunobiogram; KT, kidney transplantation; MPA, mycophenolate; MTP, methylprednisolone; OR, odds ratio; PBMCs, peripheral blood mononuclear cells; PCC, poor clinical course; RFUs, relative fluorescence units; ROC, receiver operating characteristic; SIR, sirolimus; STE, corticosteroids; TAC, tacrolimus.

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Conclusions: Our results highlight the potential of Immunobiogram as a tool to test the pharmacodynamic response to individual immunosuppressive drugs.

1. Introduction

Despite an improvement in short-term graft survival after kidney transplantation (KT) in recent decades, long-term graft survival remains a major obstacle to KT success [1]. Death-censored graft failure rates within 10 years of transplantation are 50.8% in the US [2] and 34.7% in Europe [3] and entail poorer quality of life for patients and high costs for healthcare systems.

After solid organ transplantation, adequate immunosuppression is crucial to prevent early acute rejection and to provide effective, long-term rejection prophylaxis [4]. Nonadherence to maintenance immunosuppression is an important risk factor for rejection and graft loss [5–7]. In clinical practice, treatment is adjusted empirically by clinicians following clinical guidelines, based on the individual's rejection-risk profile, time since transplant, and drug plasma levels, or is altered in response to kidney dysfunction, donor-specific anti-HLA antibodies (DSA), histologic evidence of rejection, malignancies, or infection [8,9]. Often, a given strategy can lead to either insufficient immunosuppression (resulting in rejection) or overimmunosuppression (resulting in opportunistic infections, malignancies, and toxicity) [4]. Diagnostic tools to help clinicians tailor immunosuppression to individual patients therefore constitute a key unmet clinical need.

Several tests [10–14] provide information on the transplant recipient's rejection risk and overall immunosuppression status [15,16], but not on their pharmacodynamic response to specific immunosuppressive drugs, which could be useful to guide clinicians in their choice of an optimal, individualized therapeutic regimen.

The Immunobiogram (IMBG) is a novel, unique, blood-based in vitro diagnostic test that provides a pharmacodynamic readout of the immune response of individual patients to a battery of immunosuppressants commonly used in KT, including mycophenolate, tacrolimus, cyclosporine, everolimus, sirolimus, steroids, and azathioprine [17]. The pharmacological basis of the IMBG is analogous to that of the antibiogram: the two assays enable overall in-vitro profiling of sensitivity to immunosuppressive and antimicrobial drugs, respectively. The degree to which each of the individual immunosuppressant drugs tested inhibits proliferation and activation of immunologically stimulated peripheral blood mononuclear cells (PBMCs) along a drug concentration gradient is represented by a dose-response curve, from which several parameters are automatically calculated. Analysis of the results allows quantification of the sensitivity of a given patient to the immunosuppressant(s) tested. We previously used the IMBG to characterize the individual immunosuppressant response profile of 60 KT patients, providing proof of concept in a clinical setting [17].

Here, we sought to confirm those results by using the IMBG to measure the pharmacodynamic response to immunosuppressive drugs in an international cohort of KT patients undergoing maintenance immunosuppressive therapy.

1.1. Objective

Key objective was to assess the association between clinical course (rejection) and immunosuppressant sensitivity profile by comparing the IMBG parameters of patients at either extreme of the clinical course spectrum.

2. Materials and methods

2.1. Study design

The TRANSBIO study (BHP-IBG-2017-01) was an international, multicenter, observational study in KT recipients from nine reference hospitals. Approval was obtained from the corresponding ethics committee of each participating hospital before beginning the study. This trial is registered at ClinicalTrials.gov (NCT03562845). All patients provided prior written informed consent.

All study procedures fulfilled all ethics requirements of the Declaration of Helsinki and Good Clinical Practice (GCP) standards. A clinical research organization monitored the clinical study and ensured compliance with quality assurance and control systems, established standard operating procedures, GCP, and applicable regulatory requirements. Our quality management system fulfills the requirements of the Management System Standards ISO 13485.

The inclusion criteria were: patients aged 25–70 years who had undergone KT at least 1 year before inclusion.

The association between IMBG parameters and clinical outcome was assessed in 2 patient groups selected according to clinical course (renal function impairment AND signs of rejection) over the preceding 12 months (poor [PCC] versus good clinical course [GCC] (Table 1).

Patient groups were defined applying criteria commonly used in clinical practice.

The following data were collected: donor type, donor and recipient sociodemographic variables; medical history including diagnosis of native renal disease; time since transplantation; renal failure cause; immunological history before and after transplantation; relevant

Table 1
Study population selection criteria.

Patient subgroup	Inclusion criteria	Exclusion criteria
POOR CLINICAL COURSE (PCC)	Renal function deterioration in the last 18 months (increase in Cr levels ≥15% and/or proteinuria >500 mg/day or a protein:creatinine ratio > 500 mg/g or increases by ≥50%) AND Signs of immunological rejection (biopsy with rejection signs according to categories 2 or 4 of BANFF 2017) AND/OR ≥ 50% increase in strength of DSA expressed as Luminex MFI compared with previous reading, always at titers >3000 UI	Rejection of informed consent Active systemic infections that required antimicrobial treatment in the preceding 2 months Active immune-based diseases with acute outbreaks during the preceding 12 months (despite immunosuppressive treatment) Severe ischemia-reperfusion injury of current KT with delayed graft function objectively evident >20 days after transplant and/or kidney transplanted from a deceased, elderly donor (>80 years)
GOOD CLINICAL COURSE (GCC)	Stable renal function in the last 12 months NO DSA titers No history of previous rejection episodes Stable immunosuppressive medication in the last 12 months*	Double transplant (renal + another organ) Active infection with HIV, HBV, or HCV or other severe infectious diseases that prevent processing of blood samples in a conventional laboratory Chronic allograft injury (CAI) apparently unrelated to immune processes (as determined by investigator) Recurrent primary kidney disease.

* (no change in corticosteroid or mycophenolate doses and changes in tacrolimus dose <20% over previous 12 months).

transplant-related clinical outcomes (mainly rejection episodes); presence of de novo DSA (dnDSA) and biopsies; and data on immunosuppressive therapy and other concomitant treatments. All participants completed an adherence questionnaire.

Routine hemogram and serum biochemistry tests were performed; erythrocyte sedimentation rate, and serum C-reactive protein determinations for assessment of systemic evidence of infection and/or inflammation; and urinalysis to determine urinary protein levels and urine protein: creatinine ratio.

2.2. Sample collection and processing

Blood samples (3 × 10-ml samples) were collected in sodium heparin tubes during routine outpatient visits to the transplantation clinic.

At each center, PBMCs were isolated using a standard Ficoll™ gradient procedure, counted, and resuspended in animal-free serum freezing media at a ratio of one cryogenic vial per blood tube. Vials were inserted into a cell-freezing container and maintained at −20 °C for one hour and − 80 °C for approximately 24 h and finally stored in liquid nitrogen. After three weeks, the frozen samples were shipped to Biohope facilities for IMBG analysis. Samples were encoded and processed in batches according to patient and time of arrival.

2.3. Immunobiogram assay

The Immunobiogram assay was performed as previously described [17]. Briefly, the IMBG plate is designed to simultaneously test two control conditions (positive control [C+], consisting of stimulated PBMCs; and blank control, without PBMCs) and the following seven immunosuppressant conditions: mycophenolic acid (MPA), cyclosporine A (CSA), tacrolimus (TAC), methyl prednisolone (MTP), sirolimus (SIR), everolimus (EVER) and azathioprine (AZA).

PBMCs were activated as previously described [17]. X-VIVO medium containing PBMCs was added to a previously prepared hydrogel solution to achieve a final concentration of 500,000 cells/ml per channel in the IMBG plate. For each assay, the IMBG plate was incubated for approximately 15 h at 37 °C and 5% CO_2 after placement of the discs loaded with the immunosuppressants of interest. No discs with drug were placed in either of the two control channels. Passive diffusion of the immunosuppressant through the hydrogel generates a concentration gradient along which the activation/proliferation of the embedded PBMCs is inhibited in a dose-dependent manner.

After incubation, resazurin solution (Presto Blue; Thermo Fisher Cat. A13261) was added to the final volume of hydrogel in each channel and the plates were incubated for 3 h (37 °C, 5% CO_2) before measuring PBMC fluorescence using a Spark® multimode microplate reader (Tecan) in fluorometric mode at 535/610 nm em/ex[17].

2.4. Data analysis

For each immunosuppressive drug, the IMBG acquires 15 sequential immunofluorescence readings along the concentration gradient in the IMBG channel [17], providing a read-out of PBMC activation/proliferation across the drug concentration gradient. Fluorescence data are acquired and analyzed using proprietary software (IMBG Software Version: 3.0), and automatically normalized to a scale of 0–1 (1 = positive control value). A dose-response curve is generated, plotting the immunosuppressant concentration gradient, normalized to a scale of 0–1 (0 = point of maximum immunosuppressant concentration, closest to the immunosuppressant disc; 1 = point of minimum immunosuppressant concentration, at the opposite end of the channel), against normalized fluorescence data (Fig. 1). The software automatically generates the equation describing the dose-response curve.

Data are expressed as the percentage of relative fluorescence units (% RFUs), determined relative to the positive control (100%) and the blank fluorometer reading (0%), determined independently for each plate.

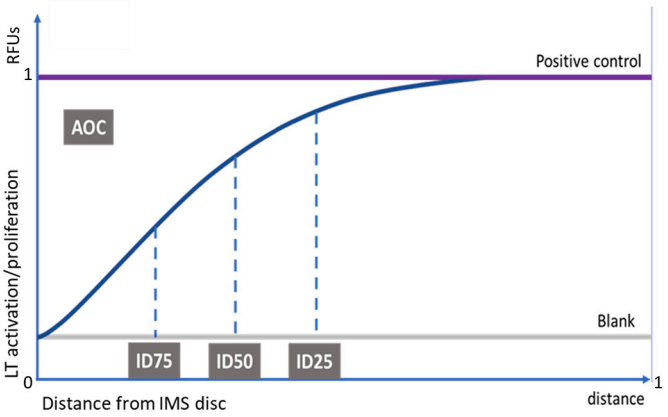

Fig. 1. Dose-response curve and key curve parameters. Dose-response curve depicting the pharmacological response to an immunosuppressant (IMS). The following curve parameters are indicated: area over the curve (AOC); 75% (ID75), 50% (ID50), and 25% (ID25) maximal inhibitory response. The x-axis represents distance, normalized to a scale of 0–1, from the point of maximum (0) to minimum (1) IMS concentration. The y-axis represents cell activity, expressed as relative fluorescence units (RFUs) normalized to a scale of 0 (blank) to 1 (positive control). For each of the aforementioned key curve parameters, higher values indicate greater sensitivity of patient T-cells to the immunosuppressant.

Our analysis focused on the following four curve parameters (Fig. 1):

- Area over the curve (AOC), i.e. the degree of inhibition of PBMCs in the presence of the immunosuppressant.
- Half-maximal (ID50), 25% maximal (ID25), and 75% maximal (ID75) inhibitory response, i.e. the points on the X-axis at which 50%, 25%, and 75% PBMC inhibition, respectively, are observed.

2.5. Statistical analysis

For descriptive analysis data were expressed as the mean, standard deviation, median, and interquartile range for quantitative frequencies and percentages for the qualitative variables.

Continuous variables were compared using the Student's *t*-test or Mann Whitney *U* test, and categorical variables using the Chi-squared test. Univariate and multivariate logistic regression assessed the association between IMBG parameters and clinical course. Based on the variables for which the univariate analysis revealed significant differences, multivariate logistic regression models generated a best-fit model and identified independent predictors associated with poor or good clinical course (dependent variable). For each sample and drug, the final model was selected by applying the parsimony principle and comparing different models based on the likelihood ratio and the results of the Wald test. The existence of confusion was determined by 10% changes in the coefficient obtained in accordance with the variables included in the model. The degree of discrimination of the models was evaluated based on the area under the curve (AUC) of receiver operating characteristic (ROC) curves. In cases in which logistic regression revealed significant results for a given immunosuppressant, the B coefficient of the slope and the variables ultimately included were used to generate an adjusted probability score. Results were expressed as odds ratio (OR) and 95% confidence interval (CI).

All analyses were 2-tailed and statistical significance was set at $p < 0.05$. Analyses were performed using SPSS and STATA SE.

3. Results

Blood samples were acquired from 145 KT patients in Europe and North America who fulfilled the inclusion criteria for the PCC or GCC

groups. Of these, 42 patients were excluded from the analysis, 25 due to a non-valid IMBG and 17 patients due to protocol deviations.

The remaining 103 patients were assigned to poor (PCC; $n = 50$) or good (GCC; $n = 53$) clinical course groups.

3.1. Patient characteristics

The demographic and clinical features and immunosuppressive treatments received by patients in the GCC and PCC groups are summarized in Table 2. In the PCC group, and according to the selection criteria, all patients exhibited signs of immunological rejection and worsening renal function parameters over the preceding 18 months. Specifically, 28 patients (56%) had a history of previous rejection episodes; 30 (60%) were positive for dnDSA, and 40 (78%) had undergone a biopsy, which revealed abnormal findings in all but one case. Antibody-mediated rejection (ABMR) changes were observed in 72.5% of biopsies and T-cell-mediated rejection (TCMR) signs in 17.5%. Mean eGFR (ml/min/1.73 m^2) was 38.25 and 61.67 in the PCC and GCC groups, respectively.

We found no differences in other classical risk factors indicators for graft rejection in the PCC versus GCC groups (donor age and type, number of HLA mismatches, and medication adherence) (Table 2). More patients had previously undergone transplantation in the PCC (24%) versus GCC (7.3%) group ($p = 0.028$). Corticosteroid use was more frequent in the PCC (96%) versus GCC (81%) group ($p = 0.029$). More patients in the PCC (94%) than the GCC (73.6%) group were receiving three different immunosuppressive drugs ($p = 0.004$).

Concomitant corticosteroid treatment in patients treated with mycophenolate or tacrolimus revealed the following: 85.9% ($n = 73$) of mycophenolate-treated patients were also receiving corticosteroids, and corticosteroid treatment was more frequent in the PCC ($n = 38$, 95%) versus GCC ($n = 35$, 77.7%) group (p = 0.029); 89.4% ($n = 76$) of tacrolimus-treated patients were also receiving corticosteroids and corticosteroid treatment was more frequent in the PCC ($n = 40$, 97.5%) versus GCC ($n = 36$, 81.8%) group ($p < 0.03$).

The percentage of patients that received induction therapy with thymoglobulin was significantly more frequent in PCC patients than in GCC patients (47% vs 12%, $p < 0.001$). Mean immunosuppressants dose (mg/day) revealed no significant differences between the GCC and PCC group, except for corticosteroid dose, that was significantly higher in PCC subgroup (6.22 vs 4.83 mg/day, $p = 0.020$) (Table 2).

We found no significant differences in mean plasma levels for tacrolimus (GCC: 6.547 and PCC: 8.235 ng/ml, $p = 0.132$ nor for everolimus (GCC: 5.733 and PCC: 5.202 ng/ml, $p = 0.843$). Differences in plasma levels of cyclosporine A were not analyzed, as they were available only for GCC patients. Plasma levels of sirolimus could not be analyzed due to lack of data.

3.2. Association between clinical course (rejection) and individual sensitivity to prescribed immunosuppressive drugs

Based on IMBG fluorescence data, dose-response curves were generated for all patients in the GCC and PCC groups ($n = 103$) depicting the individual effects of 7 distinct immunosuppressants on PBMC proliferation/activation. We compared 4 curve parameters between the GCC and PCC groups: AOC, ID50, ID25, and ID75. Higher values indicate greater PBMC sensitivity to the inhibitory effect of the immunosuppressant.

The four parameters were compared between the PCC and GCC groups for the subgroups treated with mycophenolate ($n = 85$), tacrolimus (n = 85), corticosteroids ($n = 91$), cyclosporine A ($n = 14$), and everolimus (n = 10). Patients receiving sirolimus ($n = 4$) and azathioprine ($n = 3$) were excluded from this analysis owing to the small sample size. For all drugs, mean values for each of the four parameters were higher in the GCC versus PCC group, indicating greater sensitivity to the drugs' inhibitory effect in these patients (Fig. 2). A Student's *t*-test revealed significant differences for the following variables between PCC and GCC patients: AOC ($p = 0.011$) in mycophenolate-treated patients; ID75 ($p = 0.026$), ID50 ($p = 0.016$) and ID25 ($p = 0.017$) in tacrolimus-treated patients; ID50 ($p = 0.022$) and ID25 ($p = 0.032$) in steroid-

Table 2
Characteristics of the study population.

	Good Clinical Course (n = 53)	Poor Clinical Course (n = 50)	*p* value
Recipient age, mean (SD)	52.02 (11.09)	47.54 (12.03)	0.052
Recipient sex, (% male)	29 (54.7%)	30 (60%)	0.082
Recipient age at last transplantation, mean (SD)	43.53 (12.21)	41.10 (13.84)	0.346
Years since last kidney transplantation, mean (SD)	8.42 (6.34)	6.40 (5.62)	0.691
Previous transplantation (% patients)	4 (7.3%)	12 (24%)	**0.028**
History of previous acute rejection episodes (%)	0%	28 (56%)	NA
Pre-transplant number of HLA mismatches, mean (SD)	3.59 (1.51)	4.10 (1.39)	0.458
Post-transplant de novo donor-specific antibodies (%)	0%	30 (60%)	NA
Elective biopsy (% patients)	0%	40 (78%)	NA
Abnormal biopsy		39 (98%)	NA
Biopsy findings			
Antibody-mediated rejection (ABMR)		29 (72.5%)	
T-cell-mediated rejection (TCMR)		7* (17.5%)	
Borderline changes		4 (10%)	
Interstitial fibrosis-tubular atrophy		24 (60%)	NA
Donor age, mean (SD)	44.3 (13.9)	43.9 (15.87)	0.881
Donor type (% living donors)	10 (18.9%)	7 (14%)	0.60
Blood creatinine level (mg/dl), mean (SD)	1.25 (0.28)	2.09 (0.88)	NA
Urine protein:creatinine ratio (mg/g), mean (SD)	370.79 (1044)	748.71(1048.1)	NA
eGFR (ml/min/1.73 m^2), mean (SD)	61.67 (16.41)	38.25 (15.35)	NA
Induction therapy with thymoglobulin	6(13%)	23(46%)	**0.0001**
Treatment with mycophenolate (%)	45(85%)	40(80%)	0.607
Treatment with tacrolimus (%)	44(83%)	41(82%)	1.000
Treatment with cyclosporine (%)	5(9%)	9(18%)	0.256
Treatment with corticosteroids (%)	43(81%)	48(96%)	**0.029**
Treatment with everolimus (%)	4(7%)	6(12%)	0.446
Treatment with sirolimus (%)	2(4%)	2(4%)	0.953
Treatment with azathioprine (%)	2 (4%)	1(2%)	0.593
Treatment with 2 immunosuppressants (%)	15 (28.3%)	3 (6%)	**0.004**
Treatment with 3 immunosuppressants (%)	38 (73.7%)	47 (94%)	**0.004**
Dosage mycophenolate mofetil (mg/d) (mean, SD)	1011.38 (329.87)	1232.14 (523.16)	0.165
Dosage mycophenolic acid (mg/d) (mean, SD)	742.50 (285,6)	794.62 (415,2)	0.633
Dosage tacrolimus (mg/d) (mean, SD)	3.966 (2.07)	4.451 (2.43)	0.323
Dosage cyclosporine (mg/d) (mean, SD)	185 (51.84)	150 (51.54)	0.248
Dosage corticosteroids (mg/d) (mean, SD)	4.83 (0.61)	6.22 (3.90)	**0.020**
Dosage everolimus (mg/d) (mean, SD)	2.125 (0.25)	2.083 (0.92)	0.933
Dosage sirolimus (mg/d) (mean, SD)	2 (0.0)	1 (0.71)	0.184
Dosage azathioprine (mg/d) (media, SD)	62.5	50	NA
Adherence to Treatment, mean (SD)	9.61 (0.61)	9.64 (0.72)	0.820

* 5 patients with ABMR+TCMR. Bold numbers mean statistical significant differences

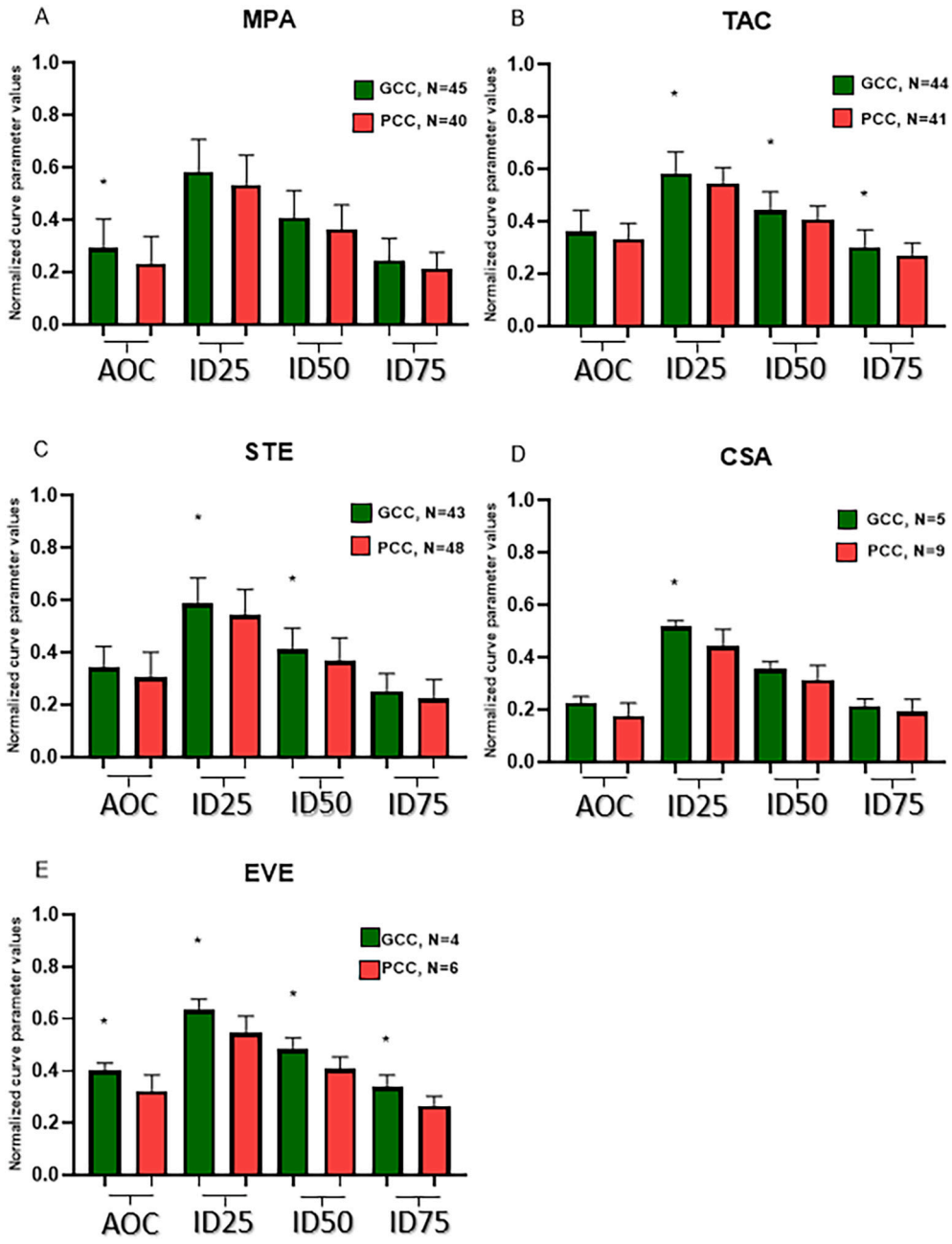

Fig. 2. Normalized dose-response curve parameters in good clinical course (GCC) and poor clinical course (PCC) groups for (A) mycophenolate (MPA, $n = 85$), (B) tacrolimus (TAC, n = 85), (C) corticosteroids (STE, $n = 91$), (D) cyclosporine (CSA, $n = 14$), and (E) everolimus (EVE, $n = 10$) treatment subgroups. Data are presented as the mean (bar) and standard deviation (error bars). *p < 0.05 versus corresponding PCC group.

treated patients; ID25 ($p = 0.033$) in cyclosporine-treated patients; and AOC (p = 0.032), ID75 ($p = 0.028$), ID50 (p = 0.033), and ID25 ($p = 0.048$) in everolimus-treated patients.

Based on the observed differences in dose-response curve parameters for individual immunosuppressants between the PCC and GCC groups, we performed univariate logistic regression analyses to evaluate the association between curve parameters and clinical course (Table 3). The following parameters were significantly associated with clinical course: AOC (OR, 0.005; CI95% <0.0001–0.353) in mycophenolate-treated patients ($p = 0.015$); ID75 (OR, <0.0001; CI95% <0.00001–0.480), ID50 (OR, <0.0001; CI95% <0.00001–0.258) and ID25 (OR, 0.001; CI95% <0.0001–0.323) in tacrolimus-treated patients ($p = 0.033$, $p = 0.021$, and p = 0.021, respectively); and ID50 (OR, 0.003; CI95% <0.0001–0.499) and ID25 (OR, 0.008; CI95% <0.001–0.735) in steroid-treated patients ($p = 0.026$, $p = 0.036$, respectively). No association was found between any curve parameters and clinical course among everolimus- or cyclosporine-treated patients, likely due to the small sample sizes (10 and 14 patients respectively).

To identify independent predictors of clinical course a stepwise multivariate logistic regression was performed for each drug based on all dose-response curve parameters for which the univariate analysis revealed significant findings, adjusting for concomitant corticosteroid treatment. For analysis of corticosteroid subgroup, the regression included concomitant intake of mycophenolate or tacrolimus as possible confounding factors. The previous transplantation variable was not included because a significant difference between the PCC and GCC groups was only observed in the tacrolimus-treated subgroup, and it showed a high degree of collinearity with concomitant corticosteroid

Table 3
Univariate logistic regression analysis: association between clinical course and immunobiogram key curve parameters.

Immunosuppressant	Curve parameters [a]	Odds Ratio	95% CI		*p*-value
			Lower Limit	Upper Limit	
MPA	AOC	0.005	<0.0001	0.353	**0.015**
(n = 85)	ID75	0.004	<0.001	2.174	0.086
	ID50	0.012	<0.0001	1.210	0.060
	ID25	0.028	<0.0001	1.192	0.062
TAC	AOC	0.002	0.0001	1.280	0.060
(n = 85	ID75	<0.0001	<0.00001	0.480	**0.033**
	ID50	<0.0001	<0.00001	0.258	**0.021**
	ID25	0.001	<0.0001	0.323	**0.021**
MTP	AOC	0.010	<0.0001	1.122	0.056
(n = 91)	ID75	0.002	<0.0001	1.155	0.056
	ID50	0.003	<0.0001	0.499	**0.026**
	ID25	0.008	<0.001	0.735	**0.036**
CSA	AOC	<0.0001	<0.00001	6.807E+2	0.096
(n = 14)	ID75	<0.0001	<0.00001	3.904E+7	0.320
	ID50	<0.0001	<0.00001	1.071E+7	0.179
	ID25	<0.0001	<0.00001	4.009E+9	0.089
EVE	AOC	<0.0001	< 0.00001	8.671E+27	0.277
(n = 10)	ID75	<0.0001	< 0.00001	1.359E+5	0.111
	ID50	<0.0001	<0.00001	4.133E+7	0.150
	ID25	<0.0001	<0.00001	7.23 E+5	0.155

Abbreviations: AOC, area over the curve; ID25, 25% maximal inhibitory response; ID50, half maximal inhibitory response; ID75%, 75% maximal inhibitory response.
[a] Key curve parameters from dose-response curves generated for patients treated with mycophenolate (MPA), tacrolimus (TAC), corticosteroids (MTP), cyclosporine (CSA), and everolimus (EVER).

treatment variable.

Immunosuppressive induction therapy with thymoglobulin was not included as a factor in the multivariate logistic regression model. Therapy with thymoglobulin is prescribed almost systematically in clinical practice to high immunological risk patients during the first 3 months after RT, but its effect lasts for no longer than 6 months. As the patients were included in the study during the maintenance phase, we don't expect that the thymoglobulin treatment still had an effect over clinical outcomes in these patients, and it could not allow us to capture the association of other meaningful variables like IMBG with rejection.

The results show a statistically significant association between clinical course and AOC and ID25, respectively, for patients treated with mycophenolate (OR 0.003, CI95% <0.001–0.258; $p = 0.01$) or tacrolimus (OR < 0.0001, CI95% <0.00001–0.202; $p = 0.016$). Among corticosteroid-treated patients, a significant association persisted between clinical course and ID50 (OR 0.003; CI95% <0.0001–0.499; $p = 0.026$). No confounding effect of concomitant mycophenolate or tacrolimus treatment was observed (Table 4).

Table 4
Results of the stepwise multivariate logistic regression analysis of parameters associated with clinical course in patients treated with mycophenolate, tacrolimus, or corticosteroids.

	Adjusted Odds Ratio[a]	95% CI		p value
		Lower Limit	Upper Limit	
MPA treatment group (n = 85)				
MPA_AOC[b]	0.003	< 0.001	0.258	**0.010**
STE	6.461	1.249	33.435	**0.026**
TAC treatment group (n = 85)				
TAC_ID25[b]	<0.0001	< 0.00001	0.202	**0.016**
STE	11.249	1.239	102.093	**0.031**
STE treatment group (n = 91)				
MTP_ID50[b]	0.003	< 0.0001	0.499	**0.026**

[a] Odds ratio after adjusting for concomitant corticosteroid therapy (STE) in patients treated with mycophenolate or tacrolimus and for concomitant mycophenolate or tacrolimus therapy in patients treated with corticosteroids.
[b] Immunobiogram key curve parameters in patients treated with mycophenolate (MPA), tacrolimus (TAC), and corticosteroids (MTP).

3.3. Analysis of probability of poor clinical course (rejection)

To assess the utility of curve parameters to predict the probability of PCC (i.e. transplant rejection), we selected the curve parameters for which the stepwise multivariate logistic regression revealed significant differences between the GCC and PCC groups: AOC for mycophenolate-treated patients; ID25 for tacrolimus-treated patients; and ID50 for corticosteroid-treated patients. Associated probabilities of PCC were calculated using the constant and the coefficient B parameters and values determined for the 10th, 25th, 35th, 50th, 65th, 75th, 90th percentiles. Bootstrap analysis performed on 3000 samples was used to calculate the CI95%. Curve parameter values were calculated for the 10th, 25th, 35th, 50th, 65th, 75th, and 90th percentiles, and a model generated in which each of these values was paired with the corresponding probability (Fig. 3). The adjusted probability indicates that the likelihood of PCC in a patient treated with a given immunosuppressant increases as sensitivity to that immunosuppressant (i.e. the curve parameter value) decreases.

To evaluate the predictive capacity of these models, an accuracy analysis was performed based on the Area Under the Curve (AUC) obtained from ROC curves, which were generated using probability values obtained from the regression analyses, obtaining following results: for mycophenolic AUC 0.721; CI95% 0.612–0.829; $p < 0.0001$; for tacrolimus AUC 0.711, CI95% 0.602–0.820, $p = 0.001$; and for corticosteroids AUC 0.632, CI95% 0.518–0.746, $p < 0.05$.

4. Discussion

The IMBG test provides a quantitative measure of the degree to which the patient's stimulated PBMCs are inhibited by a given immunosuppressive drug in vitro, based on several dose-response curve parameters. Higher values indicate greater PBMC sensitivity to a given drug.

For all immunosuppressants, mean values for all parameters were lower in the PCC (rejection) versus the GCC group, indicating lower sensitivity to the prescribed medication in patients with PCC, though this difference only reached statistically significant values for some parameters: AOC in mycophenolate-treated patients; ID75, ID50 and ID25 in tacrolimus-treated patients; ID50 and ID25 in steroid-treated patients; ID25 in cyclosporine-treated patients; and AOC, ID75, ID50,

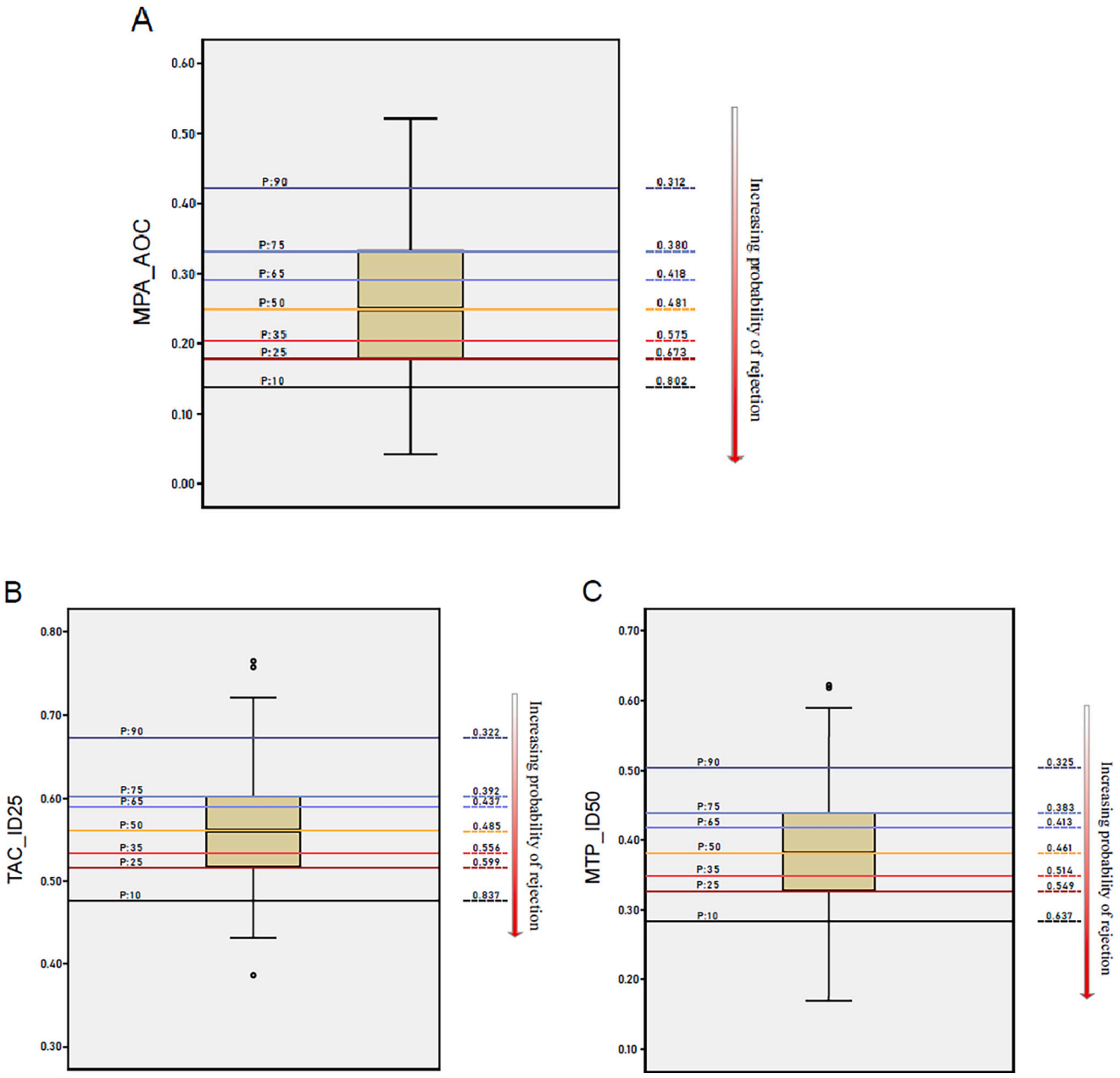

Fig. 3. Boxplots in which selected curve parameters, calculated for the indicated percentiles, are paired with the probability of poor clinical course (PCC), calculated for the corresponding percentiles based on the constant and coefficient B parameters from the multivariate logistic regression analysis. Data are shown for the following treatment subgroups and curve parameters: (A) mycophenolate (MPA), area under the curve (AOC); (B) tacrolimus (TAC), ID25; (C) corticosteroids (MTP). Y-axis represents normalized curve parameter values. In all cases, the likelihood of PCC in a given patient (depicted in the sidebar on the left) increases as sensitivity to the immunosuppressant (curve parameter values expressed for each percentile in the boxplot) decreases.

and ID25 in everolimus-treated patients. This consistent pattern, and the significant differences observed for certain curve parameters, suggest a relationship between clinical course and sensitivity to the prescribed immunosuppressive drug. Subsequent univariate logistic regression analysis confirmed an association between in vitro sensitivity (i.e. curve parameter values) and clinical course in the mycophenolate, tacrolimus, and corticosteroids treatment subgroups.

Multivariate analysis demonstrated independent associations between clinical course and AOC, ID25, and ID50 for the mycophenolate, tacrolimus, and corticosteroid treatment subgroups, respectively. These associations persisted after adjustment for concomitant treatments. Because KT patients receive combined treatment with several immunosuppressants, confounding effects on clinical outcomes of concomitantly administered drugs must be considered. This level of analysis is lacking in other studies of the effect of individual immunosuppressive drugs on patient outcomes [19–21]. These data corroborate the findings of the proof-of-concept study [17] and indicate that the patient's immune response to their prescribed drug, as measured by the IMBG, reliably correlates with the clinical outcome.

T cells are key players in adaptive immunity and chronic inflammation, and are key targets of current immunosuppressive regimens, which suppress their activity against the transplant [7,21,22,23]. Direct determination of drug targets (e.g. enzyme activity or T-cell subsets) as a pharmacodynamic surrogate may help to better assess individual responses to immunosuppressive drugs [24]. The IMBG targets patient T cells, which are the most abundant cell type in PBMCs, and quantifies the degree to which a given immunosuppressant inhibits their activation. An arguably limiting feature of the test is the non-antigen-specific

stimulation of patient PBMCs prior to exposure to the immunosuppressant in the IMBG plate. While antigen-specific stimulation may be preferable, this would require the availability of donor-specific antigens, complicating implementation of the test in clinical settings in the future, and would result in varying degrees of PBMC stimulation for each patient, potentially masking differences in the response to the drug and making response results difficult to compare between patients.

The observational, cross-sectional design of this study is common in the context of in vitro diagnostic test development and is appropriate given the limited number of relevant clinical events observed in KT patients (due to low rates of transplant rejection). Study limitations include the lack of a gold standard test to measure the pharmacodynamic response to individual immunosuppressive drugs, which precluded test validation using a reliable comparator. Our analysis thus focused on the association between immunosuppressant sensitivity and key clinical variables associated with transplant rejection.

Key strengths of this study include its international, multicenter nature and our quality management system. Moreover, patients were assigned to the PCC or GCC groups based on biopsy findings and the presence of dnDSA, which are the variables most commonly used to assess the likelihood of graft rejection in clinical practice.

Many molecular biomarkers (e.g. donor-derived cell-free DNA, RNA in blood and urine, kSORT gene expression assay) [10–14] can accurately estimate the probability of graft rejection, precluding the need for invasive biopsies. Others (e.g. ELISPOT, Immuknow) [15,16] assess the patient's global immunosuppression status. However, biomarkers that predict the pharmacodynamic response to specific drugs are currently lacking in routine clinical practice. Our results demonstrate that patient sensitivity to a given immunosuppressant is associated with clinical outcome. In the future, combination of the IMBG with existing biomarkers that predict rejection risk could aid decision-making by clinicians and facilitate optimization of immunosuppressive therapy.

In conclusion, our findings underscore the potential of the IMBG as a clinical tool to test the pharmacodynamic response to individual immunosuppressive drugs. Ongoing studies will allow evaluating its use in early post-transplantation stages, as well as changes in patient IMBG profile that occur during follow-up.

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Disclosure

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The authors based in the reference centers that participated in the study (del Mar, 12 de Octubre, La Paz, Vall d'Hebron, and Puerta de Hierro Hospitals in Spain; Wroclaw Medical University in Poland, Massachusetts General Hospital in Boston, USA; Essen University Hospital, in Germany; and Rigshospitalet University Hospital, Copenhagen, Denmark) are independent researchers who hold no shares in BIOHOPE nor have received financial compensation from Biohope. Only common expenses (materials, working hours) accrued during the research were charged to the research project financed by the European Commission.

The authors declare that all research was conducted in the absence of other third-party financial or commercial relationships that could be construed as a potential conflict of interest.

This research uses some technologies that are property of BIOHOPE and are covered by European Patent EP 17382399.8 "METHOD FOR PREDICTING AND MONITORING CLINICAL RESPONSE TO IMMUNOMODULATORY THERAPY", of which the following former and current Biohope employees are inventors: Javier Dotor de las Herrerías, Marianna Di Scala, Verónica Sánchez, Isabel Portero Sánchez.

Authorship

JP designed the clinical protocol, recruited patients and obtained the clinical samples, reviewed the statistical analyses, and contributed to and revised the final draft of the manuscript.

CJ, MK, DS, CNK, JMP, OW, SSS, AA, MC and EA helped develop the study protocol, recruited patients, and obtained the clinical samples and contributed to and revised the final draft of the manuscript.

AO, immunology specialist in Biohope's clinical laboratory, created the mathematical algorithms to analyze bioassay outputs, participated in preliminary data analysis, revised the statistical analyses, and contributed to the final draft of the manuscript.

TD compiled all data for analysis, designed, led, and revised the statistical analyses, and contributed to the final draft of the manuscript.

IP is coinventor of the immunobiogram assay, designed the clinical protocol, led the design of the mathematical algorithms used to analyze bioassay outputs, revised the statistical analyses, and contributed to the final draft of the manuscript.

Data availability

The data that has been used is confidential.

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