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ORIGINAL ARTICLE

Monitoring of intracellular adenosine triphosphate in CD4$^+$ T cells to predict the occurrence of cytomegalovirus disease in kidney transplant recipients

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SUMMARY

The measurement of intracellular concentrations of adenosine triphosphate (iATP) in phytohemagglutinin-stimulated CD4$^+$ T cells constitutes a surrogate marker for post-transplant cell-mediated immunity (CMI). This assay has shown suboptimal accuracy for predicting infection after kidney transplantation (KT). We hypothesize that its predictive capacity depends on the specific contribution of the CMI to host–pathogen interactions. We assessed iATP levels in 100 KT recipients at baseline and months 1, 3, and 6 (363 measurements). No association was found between iATP at month 1 and the risk for overall or bacterial infection, although such association was evident for cytomegalovirus (CMV) disease (multivariate-adjusted hazard ratio [per 50-unit increment]: 0.83; *P*-value = 0.048). There were no significant differences in mean iATP between stable patients (319.4 ng/ml) and those developing overall (304.1 ng/ml) or bacterial infection (346.9 ng/ml) over the 45 days following monitoring. However, iATP was significantly lower in patients who developed CMV disease (223.5 ng/ml; *P*-values <0.002). The optimal cutoff (265 ng/ml) for predicting CMV disease in patients not receiving antiviral prophylaxis yielded sensitivity, specificity, positive, and negative predictive values of 85.7%, 68.3%, 15.2%, and 98.6%, respectively. In conclusion, a non-pathogen-specific monitoring of CMI by means of iATP informs the risk of CMV disease in KT recipients.

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Key words
cytomegalovirus disease, ImmuKnow assay, immune monitoring, intracellular adenosine triphosphate, kidney transplantation, predictive value

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Introduction

Post-transplant infection constitutes a major source of morbidity and mortality after kidney transplantation (KT) [1]. The application of immune monitoring strategies has been proposed as an approach to minimize the risk of such complication by tailoring immunosuppressive therapy to individual needs [2]. Different

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biomarkers [3–7] have been used as surrogates for the overall amount of immunosuppression, although most of these strategies are limited by the lack of technical standardization and validated cutoff values [2]. In addition, certain parameters – such as the enumeration of peripheral blood lymphocyte subpopulations (PBLSs) – do not provide functional insight into the recipient's immune response [2].

The approval in 2002 by the Food and Drug Administration of a commercial assay based on the *in vitro* measurement of intracellular adenosine triphosphate (iATP) concentrations in phytohemagglutinin (PHA)-stimulated peripheral blood $CD4^+$ T cells (ImmuKnow®; Cylex™ Inc., Columbia, MD, USA) was welcomed as a significant advance to better assess the efficiency of the immunosuppressive treatment [8]. The increase in iATP constitutes a first step in the process of polyclonal expansion experienced by T cells under nonspecific stimulation [9]. By comparing healthy controls and solid organ transplant (SOT) recipients, different categories in the cell-mediated immune (CMI) response were proposed on the basis of well-defined cutoff values for iATP levels [10]. Lastly, the protocol of the ImmuKnow® assay is relatively simple and does not require special laboratory equipment.

Notwithstanding these advantages and the large number of studies performed [11–15], the clinical application of this biomarker is not widespread, likely due to the suboptimal performance shown by the ImmuKnow® assay for predicting post-transplant infection [16,17]. A shortcoming of most studies is that the indication for measuring iATP was motivated by the clinical suspicion of infection rather than being ordered within a scheduled monitoring strategy. On the other hand, the relative contribution of the CMI response to the control of infection differs according to the nature of the pathogen (i.e., extracellular bacteria or viruses) and, therefore, it is plausible that the actual predictive value of the iATP levels may have been underestimated by collapsing into a single outcome the development of any type of post-transplant infection [2].

We hypothesize that the predictive accuracy of the ImmuKnow® assay may be improved by applying a systematic scheme of monitoring throughout the first month after KT and by focusing on the occurrence of opportunistic infections in which the CMI response plays an instrumental protective role, ultimately leading to the proposal of pathogen-specific cutoff values for the iATP levels.

Subjects and methods

Study population and setting

We conducted a prospective cohort study at the University Hospital "12 de Octubre" (Madrid, Spain). From December 2011 to March 2013, all consecutive adult patients (≥18 years) undergoing KT were included in a prospective immune status assessment, as detailed below. We excluded patients with known primary immunodeficiency, human immunodeficiency virus infection with a $CD4^+$ T-cell count $<0.5 \times 10^3$ cells/μl, simultaneous pancreas–kidney transplant recipients, recipients with primary graft nonfunction, and those who died or developed graft loss requiring graft removal within the first week. The local Clinical Research Ethics Committee approved the study protocol and written informed consent was obtained from all participants.

Study design

Patients were enrolled at the moment of the transplantation and followed for a maximum of 24 months, unless death or graft loss occurred before. The minimum intended follow-up period was 12 months. All patients underwent an immune status assessment at scheduled times that included the measurement of iATP levels in peripheral blood $CD4^+$ T cells (ImmuKnow® assay) and total lymphocyte and PBLS counts. Blood samples were systematically collected at baseline (just before transplantation) and at months 1, 3, and 6. The *study outcome* was the occurrence of overall infection during the first 24 months after transplantation and, specifically, bacterial infection and cytomegalovirus (CMV) disease.

Immune function assays

Measurement of iATP levels was performed within 6 h of sampling by means of the ImmuKnow® assay according to the manufacturer's instructions [9]. PBLSs were enumerated by means of fluorescent monoclonal antibodies [18]. Detailed descriptions of both procedures are available as Supporting Information.

Immunosuppression and prophylaxis regimens

Details on immunosuppression regimens have been provided elsewhere (Supporting Information) [3,4]. All patients received preoperatively a single dose of intravenous (IV) cefazolin, replaced with ciprofloxacin in

those with ß-lactam hypersensitivity or with targeted prophylaxis according to antimicrobial susceptibility in those with known colonization with multidrug-resistant bacteria. Prophylaxis for *Pneumocystis jirovecii* was administered for 9–12 months with trimethoprim-sulfamethoxazole (160/800 mg 3 times weekly) or monthly IV pentamidine. Regarding CMV prevention strategies, antiviral prophylaxis with IV ganciclovir (5 mg/kg daily) followed by oral valganciclovir (900 mg daily, with dose adjusted for renal function) was scheduled for 6 months in the presence of donor/recipient mismatch (i.e., the seronegative recipient of an organ from a seropositive donor [D+/R−]). Seropositive patients (R+) undergoing induction therapy with antithymocyte globulin (ATG) were scheduled to receive CMV antiviral prophylaxis for 3 months [19,20]. Preemptive therapy in intermediate-risk (R+) patients was not systematically performed during the study period.

Definitions

The diagnosis of *post-transplant infection* was established if one or more of the following criteria were met: (i) a positive culture of an unequivocally pathogenic microorganism from any sample; (ii) the isolation of any microorganism from a sample obtained under sterile conditions; (iii) the isolation of a potentially pathogenic microorganism from any sample accompanied by clinical symptoms of infection; and (iv) clinical data suggestive of infection without microbiological isolation and complete resolution under antimicrobial treatment. Febrile episodes were excluded if no causative agent was isolated and no antimicrobial treatment was needed to obtain clinical resolution. Episodes of asymptomatic bacteriuria and lower urinary tract infection were excluded from the definition of bacterial infection. Opportunistic infection was defined as that due to predominantly intracellular bacteria (i.e., mycobacteria), herpesviruses, yeasts, and molds [3,21]. *CMV disease* included *viral syndrome* and *end-organ disease* [22]. Asymptomatic episodes of CMV, Epstein–Barr virus (EBV), or BK polyomavirus (BKPyV) infection were not analyzed. Further definitions used in the study [23–25] are detailed as Supporting Information.

Statistical analysis

Quantitative data were shown as the mean ± standard deviation (SD) or the median with interquartile ranges (IQR). Qualitative variables were expressed as absolute and relative frequencies. Categorical variables were compared using the chi-square test, Fisher's exact test, or McNemar test for repeated measures, whereas Student's *t*-test or Mann–Whitney *U*-test was applied for continuous variables. Pearson's correlation coefficients were calculated to assess the linear associations between normally distributed variables.

The predictive value of iATP was studied by two different approaches. First, iATP levels measured at the different time points were categorized into three different groups according to conventional cutoff values established in the literature: low (≤225 ng/ml), moderate (225–525 ng/ml), and strong CMI responses (≥525 ng/ml) [10]. Cumulative incidence curves were constructed for study outcomes (overall and bacterial infection and CMV diseases) with death treated as a competing risk, and differences between groups were compared with the log-rank test. Uni- and multivariate Cox regression models were used to evaluate the association between iATP categories at each point (baseline and months 1 and 6 after transplantation) and outcomes throughout the following periods (early [first month], intermediate [months 1–6], and late periods [months 6–24], respectively) [1]. Associations were expressed as hazard ratios (HRs) and 95% confidence intervals (CIs).

In a second set of analysis, iATP levels were considered as a continuous variable and compared according to the occurrence of an infectious event within the first 45 days following each monitoring point. Such temporal cutoff was chosen to maximize the biological plausibility of the impact of iATP levels on the risk of infection by minimizing the confounding effect of other variables (i.e., changes in immunosuppression levels) [14]. Patients were labeled as stable when no infection or rejection was diagnosed during the 45-day period. The corresponding monitoring point was excluded from analysis if both rejection and infection occurred within the following 45 days, due to the difficulty to precisely assess the causal relation between both events. Areas under receiver operating characteristic (auROC) curves were constructed to test the performance of iATP values in predicting the occurrence of infection. The optimal cutoff value with the highest value for the combined sensitivity and specificity was identified by means of the Youden's index (J statistic = Sensitivity + Specificity − 1) [26]. In an attempt to correct for the potential overoptimism of such an approach, we performed a further sensitivity analysis by randomly splitting the data into a training and an independent testing set and by recalculating the diagnostic performance of the selected cutoff value for iATP level in each of these samples.

All the significance tests were two-tailed. Statistical analysis was performed using SPSS v. 20.0 (Statistical Package for Social Sciences Inc., Chicago, IL, USA) and graphics were generated with PRISM v. 6.0 (GraphPad Software Inc., La Jolla, CA, USA).

Results

Baseline characteristics and post-transplant outcomes

We included 100 patients whose clinical characteristics are detailed in Table 1. The median follow-up period was 564.5 days (IQR: 464.0–646.8). Twenty-six patients (26.0%) experienced at least one episode of acute graft rejection, with a median interval between transplantation and the first episode of 51.5 days (IQR: 15.8–101.8). Out of 25 biopsy-proven episodes, 15 (60.0%) were classified as acute cellular rejection, 5 (20.0%) as acute humoral rejection, and 5 (20.0%) as mixed cellular and humoral rejection. Four patients (4.0%) were diagnosed with chronic rejection after a median interval of 343.0 days (IQR: 191.3–660.5). Regardless of the scheduled duration of prophylaxis, (val)ganciclovir was actually given for a mean of 5.4 months and 79.7% (47/59) of patients completed the planned regimen. 1-year survival rate was 96%, and three patients (3.0%) died at a median interval of 371.0 days after transplantation.

Post-transplant infection

Overall, 53 patients (53.0%) experienced 120 episodes of infection (incidence rate: 2.23 per 1000 transplant-days). Most of them were bacterial (81 [67.5%]), followed by viral (30 [25.0%]) and fungal (9 [7.5%]) infections. The most commonly isolated bacteria were *Klebsiella pneumoniae* (28.4%) and *Escherichia coli* (17.3%). On the other hand, CMV was the most common viral agent, with 22 episodes of disease (13 cases of viral syndrome and 9 cases of probable colitis [one of them associated to hepatitis]) diagnosed in 19 patients after a median interval from transplantation of 101.5 days (IQR: 56.0–177.3). The distribution of clinical syndromes is detailed as in Table S1.

Immune monitoring and kinetics of iATP levels

A total of 363 measurements of iATP levels were performed (median of 3.6 ± 0.7 monitoring points per patient). All patients had baseline measurement, whereas monitoring at months 1, 3, and 6 after transplantation was performed in 86, 84, and 70 patients, respectively. The median intervals from transplantation to each of these points were 38.5 ± 11.7, 100.8 ± 20.6, and 209.2 ± 59.5 days. In addition, 23 patients had an additional monitoring point beyond month 6 (at a median interval of 319.9 ± 69.5 days). There were no significant differences between patients with <3 or ≥3 monitoring points in their baseline characteristics or occurrence of graft rejection or infection (data not shown).

The distribution of patients according to the status of their CMI response at each monitoring point is detailed in Table 2. The proportion with patients with low CMI responses significantly increased from baseline to month 6 (McNemar test P-value = 0.011).

With regard to the clinical factors that influence the kinetics of iATP levels, we found that patients receiving pretransplant immunosuppression were more likely to have a low CMI response at baseline compared to the rest of the cohort (50.0% [4/8] vs. 16.3% [15/92]; P-value = 0.020). The proportion of patients with low CMI response at month 3 was also higher among those treated with ATG as induction therapy (44.2% [23/52] vs. 21.9% [7/32]; P-value = 0.038) and those diagnosed with acute rejection in the previous months (63.6% [7/11] vs. 31.5% [23/73]; P-value = 0.038).

All patients had measurements of PBLS counts at baseline. Whole blood samples at months 1, 3, and 6 were available in 96, 87, and 83 patients, respectively. There was a significant (albeit poor) correlation between iATP levels and $CD4^+$ T-cell counts at baseline (Pearson's r = 0.311; P-value = 0.002), month 3 (r = 0.468; P-value <0.001), and month 6 (r = 0.257; P-value = 0.039). We only found a significant correlation between iATP levels and $CD8^+$ T-cell counts at month 3, although the strength of the association was again low (r = 0.247; P-value = 0.031) (remaining data not shown).

Finally, there was no correlation between iATP levels and the trough blood concentrations of tacrolimus or mycophenolate mofetil at any of the analyzed monitoring points (data not shown).

Risk of infection across different categories of CMI response

We compared the occurrence of post-transplant infection according to different categories of CMI response [10]. Due to the relatively low number of episodes, events occurring between months 1 and 6 (i.e., intermediate period) were analyzed jointly. We found no differences in the cumulative incidence of infection at the

Table 1. Baseline and clinical characteristics in the study cohort (n = 100).

Variable	
Age of recipient, years [mean ± SD]	52.9 ± 16.9
Gender (male) [*n* (%)]	59 (59.0)
Pretransplant chronic comorbidities [*n* (%)]	
Diabetes mellitus	33 (33.0)
Heart disease	14 (14.0)
Chronic lung disease	10 (10.0)
Peripheral arterial disease	3 (3.0)
Chronic liver disease	4 (4.0)
Pretransplant immunosuppressive therapy [*n* (%)]*	8 (8.0)
Previous solid organ transplantation [*n* (%)]	19 (19.0)
≥2 previous transplants	2 (2.0)
Etiology of end-stage renal disease [*n* (%)]	
Diabetic nephropathy	23 (23.0)
Glomerulonephritis	22 (22.0)
Polycystosis	15 (15.0)
Nephroangiosclerosis	8 (8.0)
Chronic interstitial nephropathy	8 (8.0)
Congenital nephropathy	4 (4.0)
Reflux nephropathy	3 (3.0)
Unknown	8 (8.0)
Other	9 (9.0)
Baseline serostatus [*n* (%)]	
Hepatitis B virus (positive anti-HBc IgG)	18 (18.0)
Hepatitis C virus	2 (2.0)
CMV status D+/R+	76 (76.0)
CMV status D−/R+	16 (16.0)
CMV status D+/R−	8 (8.0)
CMV status D−/R−	0 (0.0)
Pretransplant renal replacement therapy [*n* (%)]	96 (96.0)
Hemodialysis	82 (82.0)
Continuous ambulatory peritoneal dialysis	14 (14.0)
Dialysis vintage, days [median (IQR)]	576 (300–1 081)
Age of donor, years [mean ± SD]	52.4 ± 16.4
Type of donor [*n* (%)]	
DBD donor	54 (54.0)
DCD donor	46 (46.0)
Number of HLA mismatches [median (IQR)]	5 (4–5)
Cold ischemia time, hours [mean ± SD]	16.3 ± 6.2
Induction therapy [*n* (%)]	
None	13 (13.0)
ATG	59 (59.0)
Basiliximab	28 (28.0)
Primary immunosuppression scheme [*n* (%)]	
Tacrolimus, mycophenolate mofetil, and steroids	85 (85.0)
Tacrolimus, azathioprine, and steroids	13 (13.0)
CMV antiviral prophylaxis [*n* (%)]	59 (59.0)
Scheduled for 3 months	51 (51.0)
Scheduled for 6 months	8 (8.0)
Post-transplant complications [*n* (%)]	
Need for dialysis within the first week	65 (65.0)
Renal artery stenosis	14 (14.0)
De novo post-transplant diabetes mellitus	9 (9.0)
≥1 episode of acute graft rejection†	26 (26.0)
2 episodes	2 (2.0)
Overall patient mortality [*n* (%)]	3 (3.0)
Infection-related mortality	1/3 (33.3)
Death-censored graft loss [*n* (%)]	5 (5.0)

ATG, antithymocyte globulin; CMV, cytomegalovirus; D, donor; DBD, donation after brain death; DCD, donation after circulatory death; HLA, human leukocyte antigen; IQR, interquartile range; KT, kidney transplant; R, recipient; SD, standard deviation.

*Includes chronic immunosuppressive therapy (seven patients) and HIV infection (one patient).

†Antirejection treatment consisted of steroid boluses (26 episodes), intravenous polyclonal immunoglobulins (nine episodes), plasmapheresis (eight episodes), rituximab (three episodes), and ATG (two episodes), either alone or in combination.

end of the early period (i.e., first month) across categories of CMI response at baseline (Table 3). On the opposite, the cumulative incidences at the end of the intermediate period (i.e., months 1–6) of overall, opportunistic, and bacterial infection were higher in patients with low CMI response at month 1, with the difference almost reaching statistical significance for bacterial infection (P-value = 0.058). Of note, we found a clear risk gradient for CMV disease across different categories of iATP, with cumulative incidences of 35.3%, 14.0%, and 0.0% in patients with low, moderate, and strong CMI responses, respectively (P-value = 0.012). A detailed description of the 13 cases of CMV disease diagnosed in the intermediate period in which iATP levels at month 1 were available is provided as Supporting Information (Table S2). Finally, there were no significant differences in the incidence of infection at the end of the late period (i.e., months 6–24) according to the CMI response at month 6.

The impact of iATP levels on the risk of infection throughout each post-transplant period was further explored by means of cumulative incidence analysis. There were no significant differences in cumulative incidence curves for overall or bacterial infection during the early or late periods according to the CMI responses at baseline and month 6, respectively (data not shown). When focused on the intermediate period, there were no significant differences in cumulative incidence curves

Table 2. Distribution of CMI responses in the study cohort at each monitoring point.

	Monitoring point (number of patients)				
CMI response [number of patients (%)]	Baseline (100)	Month 1 (86)	Month 3 (84)	Month 6 (70)	>6 months (23)
Low (iATP ≤225 ng/ml)	19 (19.0)	17 (19.8)	30 (35.7)	30 (42.9)	11 (47.8)
Moderate (iATP 225–525 ng/ml)	70 (70.0)	50 (58.1)	45 (53.6)	35 (50.0)	12 (52.2)
Strong (iATP ≥525 ng/ml)	11 (11.0)	19 (22.1)	9 (10.7)	5 (7.1)	0 (0.0)

CMI, cell-mediated immunity; iATP, intracellular adenosine triphosphate.

Table 3. Occurrence of post-transplant infection according to different categories of CMI response (as assessed by iATP levels) at different monitoring points.

	CMI response (iATP levels) at baseline			
Early period (first month) [number of patients (%)]	Low (≤225 ng/ml) (*n* = 19)	Moderate (225–525 ng/ml) (*n* = 70)	Strong (≥525 ng/ml) (*n* = 11)	*P*-value
Overall infection*	4 (21.1)	17 (24.3)	3 (27.3)	0.924
Bacterial infection*	4 (21.1)	13 (18.6)	2 (18.2)	0.968
Opportunistic infection	1 (5.3)	1 (1.4)	0 (0.0)	0.503
All-cause mortality	0 (0.0)	0 (0.0)	0 (0.0)	1.000
Death-censored graft loss	1 (5.3)	1 (1.4)	0 (0.0)	0.503
Lost to follow-up	0 (0.0)	0 (0.0)	0 (0.0)	1.000

	CMI response (iATP levels) at month 1			
Intermediate period (months 1–6) [number of patients (%)]	Low (≤225 ng/ml) (*n* = 17)	Moderate (225–525 ng/ml) (*n* = 50)	Strong (≥525 ng/ml) (*n* = 19)	*P*-value
Overall infection*	8 (47.1)	13 (26.0)	5 (26.3)	0.241
Bacterial infection*	7 (41.2)	7 (14.0)	5 (26.3)	0.058
Opportunistic infection	6 (35.3)	7 (14.0)	2 (10.5)	0.090
CMV disease	6 (35.3)	7 (14.0)	0 (0.0)	0.012
All-cause mortality	0 (0.0)	0 (0.0)	1 (5.3)	0.168
Death-censored graft loss	0 (0.0)	1 (2.0)	0 (0.0)	0.695
Lost to follow-up	0 (0.0)	1 (2.0)	0 (0.0)	0.695

	CMI response (iATP levels) at month 6			
Late period (months 6–24) [number of patients (%)]	Low (≤225 ng/ml) (*n* = 30)	Moderate (225–525 ng/ml) (*n* = 35)	Strong (≥525 ng/ml) (*n* = 5)	*P*-value
Overall infection*	5 (16.7)	8 (22.9)	2 (40.0)	0.479
Bacterial infection*	3 (10.0)	6 (17.1)	2 (40.0)	0.221
Opportunistic infection	2 (6.7)	2 (5.7)	1 (20.0)	0.506
All-cause mortality	0 (0.0)	1 (2.9)	1 (20.0)	0.046
Death-censored graft loss	1 (3.3)	0 (0.0)	0 (0.0)	0.526
Lost to follow-up†	2 (6.7)	1 (2.9)	0 (0.0)	0.697

CMI, cell-mediated immunity; CMV, cytomegalovirus; iATP, intracellular adenosine triphosphate.

*Lower urinary tract infection (i.e., cystitis) was not included.

†Within the first 12 months following transplantation.

across CMI categories at month 1 (Fig. 1a [log-rank test *P*-value = 0.190]), whereas some trend was found for bacterial infection (Fig. 1b [log-rank test *P*-value = 0.085]). However, a clear risk gradient according to iATP levels was only evident for CMV disease, with cumulative incidences at the end of the intermediate period of 35.3%, 12.4%, and 0.0% for low, moderate, and strong CMI responses, respectively (Fig. 1c [log-rank test *P*-value = 0.008]).

Next, the impact of CMI response on the risk of bacterial infection and CMV disease during the intermediate period was evaluated by means of separate Cox models (Table 4). Neither univariate nor multivariate analyses (HR [per 50-unit increment]: 0.95; 95% CI: 0.81–1.11; *P*-value = 0.518) revealed that iATP levels at month 1 had any impact on the risk of bacterial infection. In contrast, such association was evident for CMV disease, with iATP levels acting as an independent predictor (HR [per 50-unit increment]: 0.83, 95% CI: 0.66–0.99; *P*-value = 0.048) after adjusting for the use of antiviral prophylaxis, graft function, and previous rejection. Although there were no episodes of CMV disease during the intermediate period among D+/R− patients (as detailed in Table S2), we performed a sensitivity analysis restricted to R+ patients in which this result remained unchanged (Table S3). We also performed a second sensitivity analysis by excluding those patients who received antiviral prophylaxis. The pattern of association between iATP levels and CMV disease was similar, although the HRs were presented with wide CIs due to the low number of patients analyzed (n = 43) (Table S4).

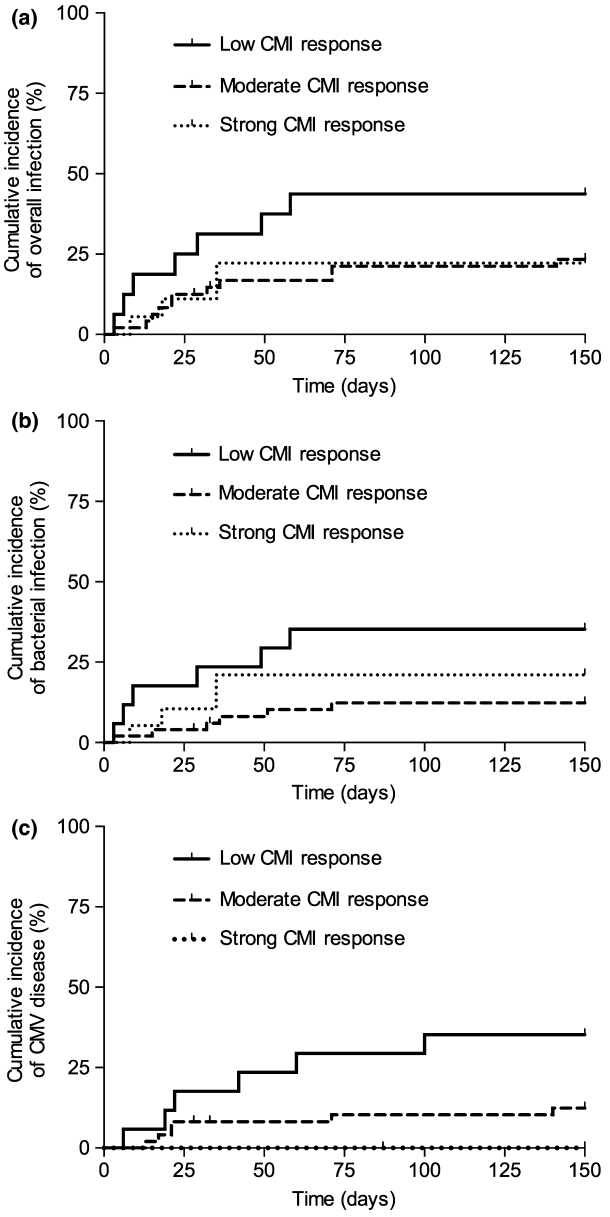

Figure 1 Cumulative incidence curves (with death as a competing risk) during the intermediate period (months 1–6 after transplantation) according to different categories of CMI response as assessed by iATP levels at month 1: (a) overall infection (log-rank test *P*-value = 0.190); (b) bacterial infection (log-rank test *P*-value = 0.085); and (c) CMV disease (log-rank test *P*-value = 0.008). CMI, cell-mediated immunity; CMV, cytomegalovirus; iATP, intracellular adenosine triphosphate.

Predictive value of iATP levels as continuous variable

Finally, we considered iATP levels as a continuous variable that was compared according to the occurrence or not of different events within the first 45 days after monitoring. As shown in Fig. 2a, iATP did not significantly differ between stable patients (i.e., event-free) (319.4 ± 158.7 ng/ml [283 monitoring points]) and those experiencing overall infection (304.1 ± 140.7 ng/ml [52 monitoring points]; *P*-value = 0.518). On the other hand, iATP levels were higher in patients that developed acute rejection (415.8 ± 176.2 ng/ml [16 monitoring points]; *P*-value = 0.011). However, clear differences emerged when the different types of infection were analyzed separately, since iATP in patients diagnosed with CMV disease (223.5 ± 100.4 ng/ml [18 monitoring points]) was significantly lower compared to either stable patients (*P*-value = 0.002) or those with bacterial infection (346.9 ± 141.4 ng/ml [34 monitoring points]; *P*-value = 0.001) (Fig. 2b). The number of episodes of invasive fungal disease or non-CMV viral infection was too low to allow separate analysis. Again, these results were similar in a sensitivity analysis restricted to R+ patients (Figure S1).

The auROC curves of iATP for predicting the occurrence of overall and bacterial infection within the

Table 4. Cox regression models for the occurrence of bacterial infection and CMV disease in the intermediate period (months 1–6 after transplantation).

	Univariate analysis			Multivariate analysis		
	HR	95% CI	*P*-value	HR	95% CI	*P*-value
Bacterial infection*,†						
Female gender	2.54	0.98–6.56	0.054	3.87	1.28–11.74	0.017
Pretransplant chronic liver disease	6.60	1.89–22.96	0.003	4.89	1.16–20.66	0.031
Acute graft rejection during the first month	4.08	1.45–11.49	0.008	3.19	1.09–9.31	0.034
iATP levels at month 1, ng/ml (per 50 ng/ml increase)	0.91	0.82–1.02	0.106	0.95	0.81–1.11	0.518
CMV disease‡						
Age of recipient, years (per unitary increment)	1.06	1.02–1.11	0.006	1.08	1.01–1.16	0.020
CMV antiviral prophylaxis for ≥3 months	0.22	0.06–0.76	0.019	–	–	–
Acute graft rejection during the first month	5.27	1.79–15.50	0.003	10.30	1.83–58.02	0.008
eGFR at month 1, ml/min (per 10-unit increment)	0.72	0.54–0.96	0.027	–	–	–
iATP levels at month 1, ng/ml (per 50-unit increment)	0.81	0.68–0.95	0.009	0.83	0.66–0.99	0.048

CI, confidence interval; CMV, cytomegalovirus; D, donor; eGFR, estimated glomerular filtration rate; HR, hazard ratio; iATP, intracellular adenosine triphosphate; R, recipient.

*Lower urinary tract infection (i.e., cystitis) was not included.

†Fifty episodes of bacterial infection were diagnosed in 18 patients during the intermediate period.

‡Thirteen episodes of CMV disease were diagnosed in 13 patients during the intermediate period.

45 days following monitoring were 0.542 (95% CI: 0.461–0.624) and 0.517 (95% CI: 0.423–0.611), respectively. In comparison, the corresponding curve for CMV disease was 0.707 (95% CI: 0.611–0.804). We selected a cutoff value of 265 ng/ml as that with the highest Youden's index, yielding sensitivity, specificity, positive, and negative predictive values of 72.2%, 65.5%, 10.2%, and 97.8%, respectively. The corresponding point estimates for the training (n = 200 monitoring points) and the testing sets (n = 151 monitoring points) were essentially similar (Table 5). Finally, when only those iATP values assessed at points at which the patient was not receiving antiviral prophylaxis were considered (n = 225), the auROC curve increased to 0.786 (95% CI: 0.695–0.878) and the prognostic performance of the test sensibly improved (Table 5).

Discussion

The results of the present study suggest that the role of monitoring iATP levels by means of the ImmuKnow® assay to predict the occurrence of post-transplant infection critically depends on the type of the causative agent. The predictive value of iATP – analyzed either as categories of CMI response [10] or continuous variable – was neglectable when overall or bacterial infection was analyzed. In contrast, the presence of a low CMI response (iATP ≤225 ng/ml) at month 1 predicted CMV disease-free survival between month 1 and 6. In addition, the iATP levels in patients that remained clinically stable over the 45 days following monitoring significantly differed from those who developed CMV disease, but not from those with bacterial infection. Therefore, and even by considering the nonspecific mitogenic stimulus used, scheduled measurements of iATP may act as a valid surrogate of the CMV-specific response after KT.

Most of previous studies on the monitoring of iATP have analyzed the occurrence of overall post-transplant infection as a single outcome, regardless of the nature of the causative agent or the clinical syndrome. By using this methodological approach, the ability of the ImmuKnow® assay in predicting infection has been found to be modest at best. A meta-analysis that summarized eight studies comprising over 1 700 patients reported a sensitivity and specificity of 58% and 69%, respectively [16]. These pooled estimates were slightly better in a second meta-analysis restricted to liver transplant recipients [17].

In addition to the originally intended role of the ImmuKnow® assay as an instrument to analyze the efficiency of post-transplant immunosuppressive regimen, it is assumed that the production of iATP by PHA-stimulated $CD4^+$ T cells recapitulates the functional responsiveness of the cell-mediated response [2,9]. However, the protective immunity to extracellular bacteria mainly depends on complement activation, antibody production, and opsonophagocytosis [4,27]. Thus,

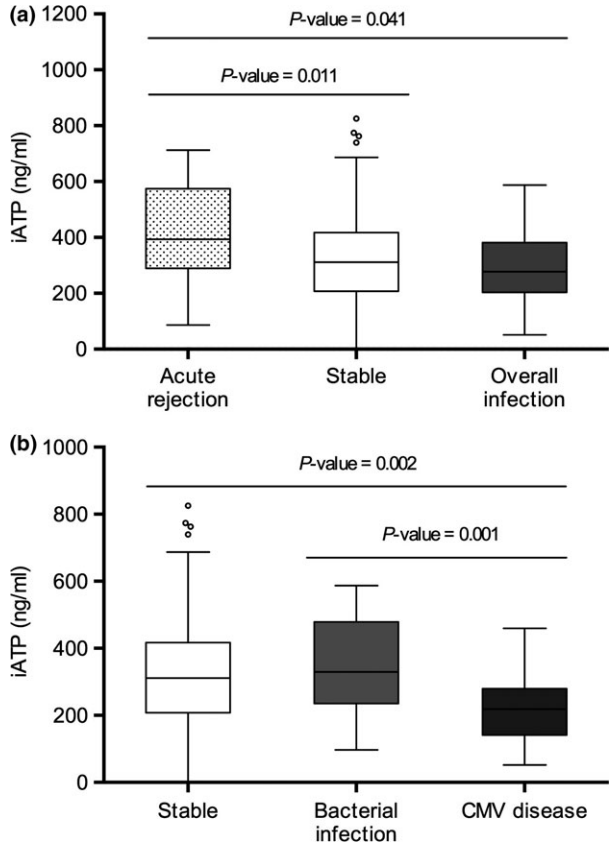

Figure 2 Tukey box and whisker plots showing iATP levels according to the occurrence of different events within the 45 days following the monitoring point: (a) comparison between patients with acute graft rejection, overall infection, and stable situation (i.e., no event); (b) comparison between patients with bacterial infection, CMV disease, and stable situation. The horizontal line within the boxes represents the median, the outer horizontal lines of the boxes are the 25th and 75th quartiles, the horizontal lines of the whiskers are the Tukey inner fences, and the closed circles represent outliers. CMV, cytomegalovirus; iATP, intracellular adenosine triphosphate.

it is plausible that the iATP concentrations may have a better correlation with the specific risk of infection due to intracellular pathogens like CMV. Accordingly, some authors have observed lower iATP levels in recipients with asymptomatic CMV o BKPyV viremia compared to stable patients [12,28–30]. Husain *et al.* explored the dynamics of iATP in lung transplant recipients according to the type of infectious syndrome. An increasing gradient in iATP levels was found across patients with CMV disease, bacterial pneumonia, and invasive fungal disease, mirroring the relative contribution of the CMI response to the immune control of each pathogen [14]. Our findings reinforce this notion. A limitation of some of these studies relies on the fact that the indication for testing was triggered by the clinical suspicion of infection, hindering the attribution of causality as the infection itself may promote T-cell exhaustion with a subsequent decrease in iATP levels [12,28,29]. In contrast, we followed a scheduled strategy for monitoring and exclusively assessed the predictive value of iATP levels with regard to the development of infection over the next weeks.

An expanding repertoire of methods that measure *in vitro* release of interferon (IFN)-γ following specific antigen stimulation is currently available for the functional characterization of the CMV-specific CMI [2,31]. Despite the promising results reported [32–34], the clinical implementation of these assays has been limited so far. Our results suggest that non-pathogen-specific functional monitoring after PHA stimulation may offer an easier alternative approach to inform the risk of CMV disease. In a previous study in which the CMV-specific CMI response was assessed with the QuantiFERON-CMV assay, Manuel *et al.* [32] observed that those with

Table 5. Performance of a cutoff value for iATP levels of 265 ng/ml for predicting the occurrence of CMV disease within the first 45 days following the monitoring point.

Setting	Sensitivity (%) (95% CI)	Specificity (%) (95% CI)	PPV (%) (95% CI)	NPV (%) (95% CI)
All monitoring points (n = 351)*	72.2 (46.5–90.1)	65.5 (60.1–70.6)	10.2 (5.5–16.7)	97.8 (94.9–99.3)
No CMV antiviral prophylaxis at the time of monitoring (n = 225)	85.7 (57.2–98.2)	68.3 (61.5–74.5)	15.2 (8.1–25.0)	98.6 (95.1–99.8)

CI, confidence interval; CMV, cytomegalovirus; iATP, intracellular adenosine triphosphate; NPV, negative predictive value; PPV, positive predictive value.

*Point estimates for the training set (n = 200 monitoring points): sensitivity: 70.0% (95% CI: 34.7–93.3); specificity: 69.5% (95% CI: 62.4–75.9); PPV: 10.8% (4.4–20.9); NPV: 97.8% (95% CI: 93.6–99.5).

Point estimates for the testing set (n = 151 monitoring points): sensitivity: 75.0% (95% CI: 34.9–96.8); specificity: 60.1% (95% CI: 51.6–68.2); PPV: 9.5% (3.6–19.6); NPV: 97.7% (95% CI: 92.0–94.7).

an indeterminate result in the mitogen tube (i.e., defective production of IFN-γ by peripheral blood lymphocytes upon PHA stimulation) carried the highest risk for CMV disease after prophylaxis discontinuation. By using a cutoff value for iATP levels (265 ng/ml) slightly different than that proposed in the literature [10], the presence of a low CMI response predicted in our experience the development of CMV disease within the following 45 days. It should be noted that none of the episodes of CMV disease occurred in the highest risk category (D+/R−), but in R+ patients that had been mostly managed without antiviral prophylaxis. The prognostic accuracy of iATP was improved in the subgroup of patients that were not receiving antiviral prophylaxis at the time of monitoring, with sensitivity and specificity values of 85.7% and 68.3%, respectively.

Some limitations to our study must be acknowledged. The number of events of infection was low, thus potentially compromising the stability of the multivariate models. Therefore, we were not able to perform separate analyses for other types of opportunistic infection (i.e., invasive fungal disease). The feasibility of monitoring iATP levels to individualize the risk of CMV disease should be taken with caution as different D/R serostatus categories were jointly analyzed, although our findings were consistent across different sensitivity analyses. Although it could be hypothesized that the performance of low iATP levels would be also valuable to predict the reactivation of viruses other than CMV (such as EBV or BKPyV) [12], the lack of systematic surveillance in our cohort precludes any definitive conclusion on this point. Finally, the positive predictive values observed were suboptimal (≈10–15%). Thus, the utility of a monitoring strategy based on iATP levels would mainly lie on its capacity to discriminate low-risk patients, even by assuming that the actual odds of CMV disease among those below the selected threshold would be relatively low.

In conclusion, a functional non-pathogen-specific monitoring of the CMI response based on the assessment of iATP levels in PHA-stimulated peripheral blood $CD4^+$ T cells may be useful to individualize the risk of CMV disease after KT. The excellent negative predictive value observed (98.6%) would allow the identification of those patients at a very low risk of CMV disease in which viral monitoring could be safely discontinued. On the contrary, the predictive value of this approach for overall or bacterial infection was very poor. Further studies aimed at evaluating the implementation of the ImmuKnow® assay to predict infection should take into account the role of the CMI in the recipient's immune response against pathogen and focus on specific forms of infection, such as CMV disease, as outcome.

Authorship

MAPJA, MFR, FLM and JMA: designed research. MAPJA, MFR, CA, EG, TRM, EG and RSJ: performed research. MAPJA, MFR, CA and FLM: analyzed the data. MFR: wrote the paper. EPA, AA and JMA: critically revised and completed the final draft of the manuscript.

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Conflicts of interest

The authors have declared no conflicts of interest.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article:

Data S1. Subjects and methods.

Data S2. Results:

Table S1. Clinical syndromes and causative agents involved in the 120 episodes of post-transplant infection.

Table S2. Detailed clinical characteristics of the 13 episodes of CMV disease occurring in the intermediate period (months 1–6 after transplantation) with available iATP levels at month 1.

Table S3. Cox regression model for the occurrence of CMV disease in the intermediate period: sensitivity analysis restricted to CMV R+ patients (i.e., after excluding high-risk [D+/R−] patients).

Table S4. Cox regression model for the occurrence of CMV disease in the intermediate period: sensitivity analysis restricted to patients not receiving CMV antiviral prophylaxis.

Figure S1. Tukey box and whisker plots showing iATP levels according to the occurrence of different events within the 45 days following the monitoring point in a sensitivity analysis restricted to CMV R+ patients: (a) comparison between patients with acute graft rejection, overall infection and stable situation; (b) comparison between patients with bacterial infection, CMV disease and stable situation.

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