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ORIGINAL ARTICLE

Monitoring of gene expression in tacrolimus-treated de novo renal allograft recipients facilitates individualized immunosuppression: Results of the IMAGEN study

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Aims: Calcineurin inhibitors (CNI) have a small therapeutic window, and drug monitoring is required. Pharmacokinetic monitoring does not correlate sufficiently with clinical outcome. Therefore, the expression of nuclear factor of activated T cells (NFAT)-regulated genes in the peripheral blood has been suggested as a potentially useful immune monitoring tool to optimize CNI therapy. NFAT-regulated gene expression (RGE) was evaluated in renal allograft recipients as predictive biomarker to detect patients at risk of acute rejection or infections.

Methods: NFAT-RGE (interleukin-2, interferon-γ, granular-macrophage colony-stimulating factor) was evaluated by quantitative real-time polymerase chain reaction in whole blood samples at day 7, day 14, month 1, 3, and 6 after transplantation in 64 de novo renal allograft recipients from 3 European centres. Immunosuppression consisted of tacrolimus (Tac), mycophenolic acid, and corticosteroids.

Results: Tac concentrations (C0 and C1.5) correlated inversely with NFAT-RGE ($P < .01$). NFAT-RGE showed a high interindividual variability (1–61%). Patients with high residual gene expression (NFAT-RGE ≥30%) were at the increased risk of acute rejection in the following months (35 *vs.* 5%, $P = .02$), whereas patients with low residual gene expression (NFAT-RGE <30%) showed a higher incidence of viral complications, especially cytomegalovirus and BK virus replication (52.5 *vs.* 10%, $P = .01$).

Conclusions: NFAT-RGE was confirmed as a potential noninvasive early predictive biomarker in the immediate post-transplant period to detect patients at risk of acute rejection and infectious complications in Tac-treated renal allograft recipients. Monitoring of NFAT-RGE may provide additional useful information for physicians to achieve individualized Tac treatment.

The authors confirm that Claudia Sommerer is the principal investigator for this paper and that she had direct clinical responsibility for patients.

KEYWORDS
biomarker, cytomegalovirus, pharmacodynamics, pharmacokinetics, rejection, renal transplantation, tacrolimus

1 | INTRODUCTION

Optimal immunosuppression is necessary to achieve the best transplant function and patient survival after renal transplantation. Several attempts have been undertaken to improve immunosuppressive treatment. Most of the new promising agents failed before market introduction. At this time, cell therapy to induce tolerance seems to be promising but is far from daily clinical use,[1,2] while calcineurin inhibitors (CNIs) are still the most widely used immunosuppressive agents in transplantation.[3,4] CNIs are critical dose drugs and have a large pharmacokinetic interpatient variability. Therapeutic drug monitoring is necessary to balance efficacy and toxicity due to the narrow therapeutic window. One step to an optimized immunosuppression might be an individualized CNI treatment based on adequate monitoring tools.[5,6] Pharmacokinetic monitoring is well established in clinical practice but does not reflect the biological activity of the drug. Sensitivity to CNIs varies widely between individuals due to differences in intracellular drug accumulation but more importantly, due to variable and difficult to predict genetic and epigenetic differences in the immune responsiveness. Therefore, the measurement of a CNI trough concentration in peripheral blood does not necessarily predict the biological activity of the drug in immune cells of a particular patient.

Several biomarkers have shown promising results predicting the rejection risk, graft dysfunction, and the personal response to immunosuppressive agents. Pharmacodynamic monitoring strategies have been used to directly measure the functional effects of CNIs.[7–10]

CNIs inhibit the phosphatase activity of calcineurin.[11,12] The main substrate of calcineurin in T cells is the nuclear factor of activated T cells (NFAT). This transcription factor does not translocate into the nucleus in its phosphorylated form; therefore, CNIs prevent the transcription of genes regulated by NFAT. More than 100 genes encoding a variety of cytokines (such as **interleukins [ILs]-2**,[13] 4 and 17, **interferon-γ [IFNγ]**,[14] and **tumour necrosis factor [TNF]-α**[15]) as well as several components of signal transduction pathways, cell cycle regulatory proteins, and apoptosis-controlling proteins in T cells seems to be regulated by this transcription factor.[16]

Therefore, the transcription of NFAT-regulated genes in the peripheral blood has been suggested as a potentially useful immune monitoring tool to individualize CNI therapy.[17–19] Previous studies have evaluated the residual expression of NFAT-regulated gene expression (RGE) in solid organ transplant recipients.[20,21] Most of these studies evaluated NFAT-RGE in long-term stable transplanted patients and clearly showed that strong inhibition of these genes is a sign of *oversuppression* reflected by a higher incidence of infectious (mostly viral) and malignant complications.[22–27]

What is already known about this subject

- Pharmacokinetic monitoring does not correlate sufficiently with clinical outcome.
- Pharmacodynamic monitoring provides the opportunity to individualize calcineurin inhibitor therapy.
- Monitoring of the residual expression of nuclear factor of activated T cells regulated gene expression (NFAT-RGE) reflects the biological effect of ciclosporin A treatment in long-term renal allograft recipients.

What this study adds

- Monitoring of NFAT-RGE reflects the biological effect of tacrolimus.
- NFAT-RGE is confirmed as a noninvasive early predictive marker in the post-transplant period for the risk of acute rejection and infections and allows individualized tacrolimus treatment adjustments.
- NFAT-RGE can be used as biomarker in international multicentre trials.

The aim of the blinded, prospective study was to assess the clinical utility and validate the biomarker residual NFAT-RGE in adult renal allograft recipients on tacrolimus therapy early after transplantation in 3 large European transplant centres.

2 | MATERIALS AND METHODS

2.1 | Patient cohort

De novo renal allograft recipients from 3 European transplant centres (Barcelona, Berlin, Heidelberg) were recruited in this prospective noninterventional trial (EudraCT number: 2013–001817-33). Inclusion criteria were age ≥18 years, primary renal transplant patients without other transplanted organs from deceased or living donors, willing to participate in the study, and from whom written informed consent has been obtained.[28] Exclusion criteria were recipients older than 70 years, positive for HCV, HBV or HIV panel reactive antibodies >10%, or AB0-incompatibility.

2.2 | Study design

The study included a total of 6 visits: pretransplantation, post-transplantation at day 7, and months 1, 2, 3 and 6. The patient cohort was divided into 2 groups according to the residual NFAT-RGE at day 7. The threshold of NFAT-RGE was detected by receiver operator curve (ROC) analysis in a patient cohort with a maximum time after transplantation of 6 months of an earlier evaluation in Tac treated renal allograft recipients (test cohort).[29]

At each visit safety parameters were assessed, including renal allograft function (serum creatinine, glomerular filtration rate), urinary protein, blood counts, and routine chemistry. In addition, regular monitoring of cytomegalovirus (CMV), polyomavirus (BKV) and Epstein Barr virus replication was performed. Polyomavirus viraemia was defined as BKV or John Cunningham virus levels ≥10 000 DNA copies mL^{-1} in whole blood and cytomegalovirus viraemia as CMV levels ≥1000 DNA copies mL^{-1}. Renal allograft function was assessed by calculating the glomerular filtration rate with the Modification of Diet in Renal Disease formula.

Apparent acute infections, e.g. urinary tract infections, infections of the upper or lower respiratory system, CMV infection and other herpes virus infections, were recorded. Indication biopsies were obtained when necessary. Acute rejection was diagnosed by clinical and laboratory findings and confirmed by histological evaluation of graft biopsies by an independent pathologist according to Banff 2007 criteria.[30]

The study was approved by the institutional Ethical Review Board (Ethics Committee University Hospital Heidelberg, Germany, reference number ABmu-507/2013). The study was conducted following the ethical principles of the Declaration of Helsinki and in compliance with the Good Clinical Practice Guidelines.

2.3 | Study objectives

The primary outcome was the combined endpoint consisting of biopsy-proven acute rejection (BPAR), renal allograft failure, and death. Secondary outcomes were efficacy results (treated BPAR) and safety data (renal function, infectious complications, malignancy, cardiovascular risk factors, e.g. hypertension, hyperlipidaemia, diabetes), and neurotoxic effects.

2.4 | Immunosuppressive regimen

All enrolled patients received an immunosuppressive regimen consisting of tacrolimus (Tac; Prograf, Astellas Pharma), mycophenolic acid (MPA; Myfenax, Teva Pharmaceuticals), prednisone, and basiliximab induction. The initial oral daily dose of Tac was 0.1 mg/kg and administered twice daily. The Tac dose had to be subsequently adjusted to achieve target trough concentrations of 7–10 and 5–8 ng/mL during the first and third months, respectively. MPA was administered 1 g twice daily. Steroid treatment was initiated at day 0 with 500 mg of methylprednisolone, 250 mg on day 1, 125 mg on day 2, 80 mg on day 3, 60 mg on day 4, 40 mg on day 5, and progressively decreasing the dose to reach 20 mg on day 14, and from day 15 to day 29, 15 mg of prednisone. Patients received 10 mg of prednisone from day 30 to day 60 after transplantation. After the 60th day, 5 mg/d of prednisone was administered.

Within 6 months after transplantation all patients had *Pneumocystis jirovecii* prophylaxis with cotrimoxazole. Patients with CMV-positive donors had CMV prophylaxis with valganciclovir during the first 3 months after transplantation.

2.5 | Pharmacokinetic monitoring

Tac trough concentration (C_0), Tac peak concentration ($C_{1.5}$), Tac concentration–dose ratio (Tac C/D) at day 7, and months 1, 2, 3, and 6 post-transplantation, and complete area under the curve (AUC; 0, 30 min, 1, 1.5, 2, 3, 4, 6, 8 and 12 h after drug administration) at day 7 post-transplantation were analysed. Abbreviated Tac AUC was calculated according to Miura *et al.*[31] with the term AUC_{0-12} = 7.04C0 + 1.71C2 + 3.23C4 + 15.19 and Mathew *et al.*[32] with the term AUC_{0-12} = 19.16 + (6.75C0) + (3.33C1.5).

Blood samples were collected in EDTA-K3 tubes and processed immediately. Tac concentrations in whole blood were measured by liquid chromatography/tandem mass spectrometry and MPA concentrations were measured by high-performance liquid chromatography with ultraviolet spectrometry.

2.6 | Pharmacodynamic monitoring

All samples for the evaluation of the residual expression of NFAT-RGE were analysed at the Institute of Immunology, University Hospital Heidelberg, Germany.

2.6.1 | Sample preparation

Residual NFAT-RGE was assessed in whole blood samples. Heparinized peripheral blood was stimulated with 1 mL of complete RPMI 1640 containing 100 ng/mL PMA and 5 μg/mL ionomycin (Sigma-Aldrich Corp. St. Louis, MO, USA) for 3 hours at 37°C. After red cell lysis with ACK buffer (0.15 M NH_4Cl, 1.0 mM $KHCO_3$), leucocytes were lysed with 400 μL of MagNA-Pure lysis buffer supplemented with an additional 1% (w/v) of dithiothreitol (RAS, Mannheim, Germany), and the sample was frozen at −70 °C. After thawing, mRNA was isolated with the MagNA-Pure-LC device using the mRNA standard protocol for cells. The elution volume was set to 50 μL. One aliquot of 8.2 μL RNA was reverse transcribed in a thermocycler using avian myeloblastosis virus reverse transcriptase and oligo (dT) as a primer (First Strand cDNA synthesis kit; Roche, Mannheim, Germany) according to the manufacturer's protocol. After the termination of the cDNA

synthesis, the reaction mixture was diluted to a final volume of 200 μL and stored at −20°C until polymerase chain reaction (PCR) analysis.

2.6.2 | Quantitative analysis of gene expression

The NFAT-regulated genes IL-2, IFNγ and granular-macrophage colony-stimulating factor (GM-CSF)[33] were selected for this assay from previous studies.[4,5] Gene expression was quantified using real-time (RT)-PCR with the LightCycler. Target sequences were amplified using commercially available LightCycler Primer Sets (Search-LC, Heidelberg, Germany) with the LightCyclerFastStart DNA Sybr Green I Kit (Roche, Mannheim, Germany) according to the manufacturer's protocol. The transcript concentration for the measured genes was calculated from a virtual standard curve, obtained by plotting a known input concentration of a plasmid to the PCR cycle number at which the detected fluorescence intensity reaches a fixed value. mRNA input was normalized by a constant expression value of 2 housekeeping genes (β-actin and peptidyl propyl isomerase B).

RGE after Tac intake was calculated as $C_{peak}/C_0 \times 100$, where C_0 is the adjusted number of transcripts at the Tac predose level, and C_{peak} is the number of transcripts 1.5 hours ($C_{1.5}$) after drug intake. Mean RGE was calculated as mean RGE from the 3 measured NFAT-regulated genes: IL-2, IFNγ and GM-CSF.

2.7 | Statistical analysis

Demographic data and the results of the prospective analysis were collected in a unified database. Statistical analysis was performed using SPSS software (version 26.0; Chicago, IL, USA). Data were presented as median ± standard deviation or percentages.

ROC analyses were performed to assess how well NFAT-RGE discriminated between patients at risk for the combined endpoint (death, transplant failure and acute rejection). Optimal thresholds were calculated using simultaneous maximization of sensitivity and specificity.

Frequency distributions were provided for categorical variables, and treatment groups were compared with χ^2 tests. Descriptive statistics were presented for continuous variables, and comparisons of both groups were performed with a nonparametric test (Mann–Whitney *U* test). Correlations between gene expression and Tac levels were analysed using Spearman correlation coefficient (r). All statistical tests were 2-sided and used the .05 level of statistical significance ($P < .05$).

3 | RESULTS

3.1 | Patients

In total, 64 patients were enrolled between April 2014 and June 2015 in all 3 European transplant centres (Figure 1). Transplantation was

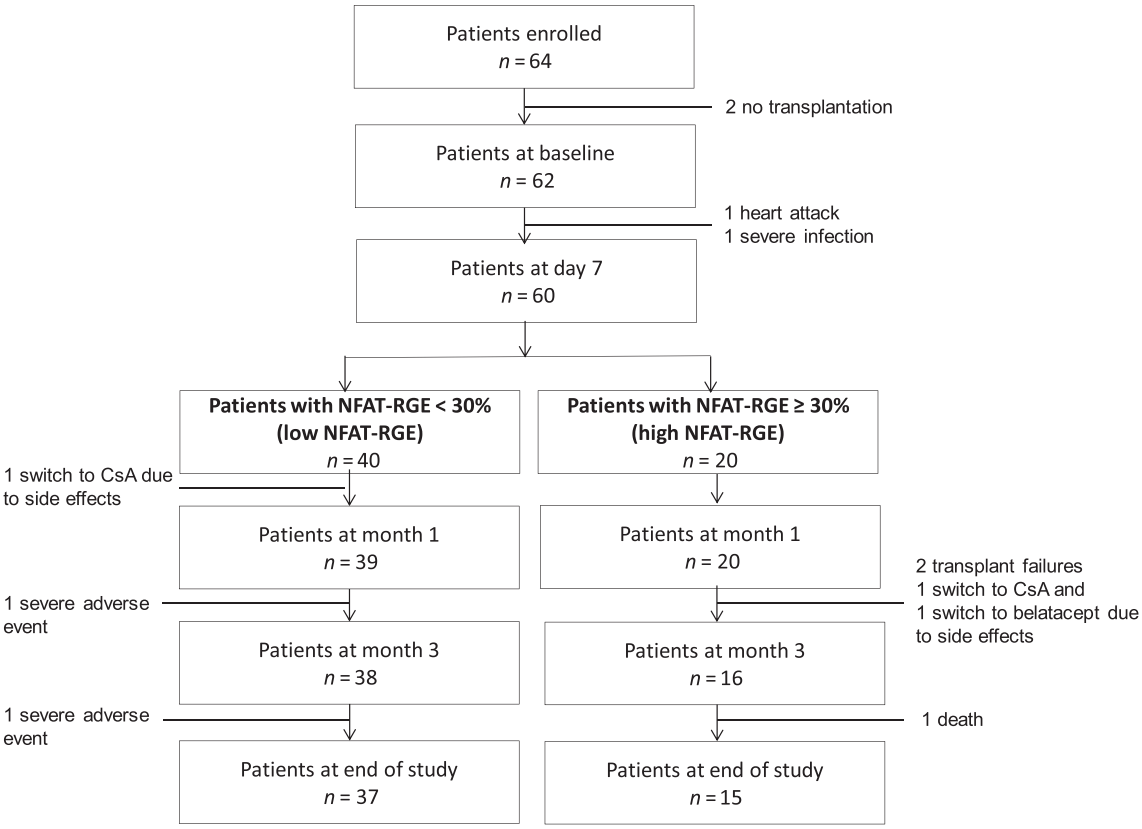

FIGURE 1 Patient flow chart

cancelled in 2 patients due to medical reasons, 1 patient suffered a heart attack and 1 other patient from a severe infection early after transplantation. Altogether, 60 de novo renal allograft recipients could be evaluated.

All, except for 3 patients with NFAT-RGE <30% and 5 patients with NFAT-RGE ≥30%, completed the study. Patient characteristics were similar between groups (Table 1).

3.2 | ROC analysis for group definition

ROC analyses for the ability of NFAT-RGE to detect patients at risk of acute rejection episodes was performed in a group of 111 renal allograft recipients with a maximum time of 6 months after transplantation of an earlier evaluation of NFAT-RGE as prognostic marker in Tac treatment.[26] Results showed a good diagnostic accuracy ($P < .01$; AUC = 0.874; 95% CI 0.759–0.989; Figure 2). Simultaneous maximization of sensitivity and specificity obtained from ROC curves was shown at NFAT-RGE of 30% (sensitivity 90.1%, specificity 85%), whereas the positive predictive value was 0.4 and the negative predictive value (NPV) was 0.99. Hit rate was 85.6%.

Consequently, we used NFAT-RGE 30% as the cut-off for further discrimination between 20 patients with low inhibition (defined as high NFAT-RGE ≥30%), and 40 patients with high inhibition defined as NFAT-RGE <30%.

3.3 | AUC

Complete AUC_{0-12} at day 7 post-transplantation could be obtained in 36 of the patients. Mean TAC AUC_{0-12} was 176 ± 81 μg*h/L. TAC AUC_{0-12} correlated significantly with abbreviated AUC calculated according to Miura ($R = .972$, $P < .001$) or Mathew (0.968, $P < .001$). TAC AUC_{0-12} and RGE correlated inversely (IL-2 RGE, $R = -.408$, $P = .017$; IFNy, $R = -,459$, $P = .006$; GM-CSF RGE, −0.499, $P = .003$; NFAT-RGE, −0.459, $P = .006$).

TABLE 1 Demographics and clinical characteristics of de novo renal allograft recipients. Data of all enrolled patients and in both study groups are shown

	All*	Low NFAT-RGE (<30%)	High NFAT-RGE (≥30%)	*P*-value
Number of patients	64	40	20	-
Recipient				
Age (y), mean ± SD	48.1 ± 12.3	47.7 ± 12.4	48.1 ± 12.7	.896
Male	41 (64.1)	24 (60%)	14 (70%)	.449
Caucasian	57 (89.1)	36 (90%)	18 (90%)	.472
BMI (kg/m^2)	25.8 ± 5.1	25.7 ± 5.0	26.1 ± 5.6	.789
ESRD leading to transplantation				.057
Glomerulonephritis	17 (26.6)	12 (30%)	3 (15%)	
ADPKD	14 (21.9)	10 (25%)	3 (15%)	
Diabetes	6 (9.4)	3 (7.5%)	3 (15%)	
Other	21 (32.8)	9 (22.5%)	11 (55%)	
Unknown	6 (9.4)	6 (15%)	0	
Transplantation				
Pre-emptive transplantation	14 (21.9%)	12 (30%)	2 (10%)	.084
Pretransplant dialysis (mo)	59.5 ± 46.7	51.2 ± 51.3	65.9 ± 42.4	.316
Cold ischaemia time (h)	9.5 ± 7.7	11.3 ± 4.6	13.6 ± 5.1	.241
Donor				
Age (y), mean ± SD	51.9 ± 12.9	51.1 ± 12.8	52.8 ± 12.3	.644
Male (%)	27 (42.2)	18 (45%)	8 (40%)	.713
Living (%)	30 (46.9)	23 (57.5%)	7 (35%)	.100
CMV- infection status donor (D) recipient (R)				.342
D−/R-	4 (6.3%)	1 (2.5%)	3 (15%)	
D−/R+	10 (15.6%)	6 (15%)	4 (20%)	
D+/R-	16 (25%)	11 (27.5%)	3 (15%)	
D+/R+	34 (53.1%)	22 (55%)	10 (50%)	

*all enrolled patients.

ADPKD, autosomal-dominant polycystic kidney disease; BMI, body mass index; D, donor; HLA, human leucocyte antigen; NFAT, nuclear factor of activated T cells; RE, residual expression; R, recipient, SD, standard deviation

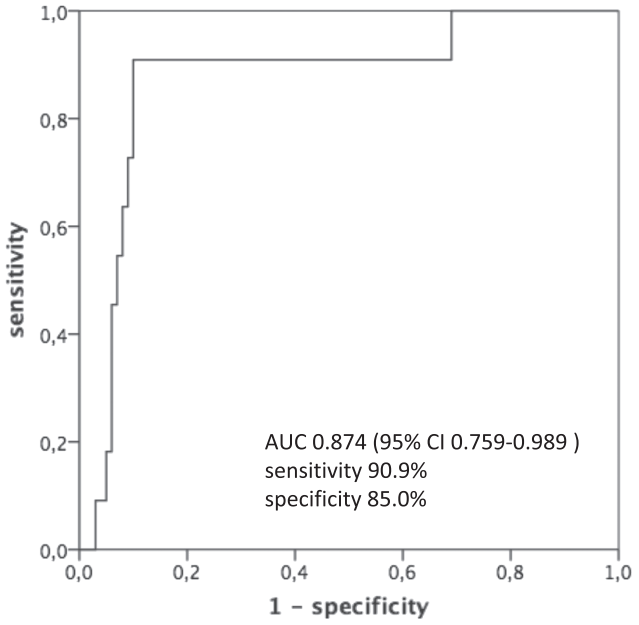

FIGURE 2 Receiver operating curve on nuclear factor of activated T cells-regulated gene expression (threshold 30%) to detect patients at risk of acute rejection episodes. Results showed a good diagnostic accuracy ($P < .01$; area under the curve [AUC] = 0.874; 95% confidence interval [CI]0.759–0.989)

3.4 | Pharmacokinetic and pharmacodynamics monitoring

Pharmacokinetic data of Tac treatment are summarized in Table 2. There was no significant difference in Tac dose, Tac C_0 or Tac C/D between patients with low (RGE <30%) and high (RGE ≥30%) residual expression of NFAT-regulated genes throughout the study period. In contrast, Tac $C_{1.5}$ was significantly higher at all time points in the patient group with residual NFAT-RGE below 30%.

NFAT-RGE and expression of all 3 genes (IL-2, IFNγ and GM-CSF) correlated inversely to the Tac levels with the highest inhibition of gene expression at the time of the peak Tac level at 1.5 hours after drug intake (day 7; NFAT-RGE 21 ± 16%, IL-2 RGE 20 ± 17%, IFNγ RGE 25 ± 18%, GM-CSF RGE 18 ± 16%). NFAT-RGE showed high interindividual variability, from 1 to 61% (Figure 3a). The pharmacodynamic marker NFAT-RGE increased from 21 ± 16% at day 7 to 34 ± 27% at month 2 in the whole patient cohort. After that, residual NFAT-RGE was stable (31 ± 25% at month 3, 39 ± 26% at month 6), but with persisting high interindividual variability ranging from 6 to 95% (Figure 3b).

Patients with an early low residual expression of NFAT-regulated genes showed significantly lower expression of all 3 genes throughout the study period (Table 2).

3.5 | Efficacy

The combined endpoint (death, graft failure and BPAR) was reached in 9 patients with a significantly higher incidence in patients with

TABLE 2 Pharmacokinetics and pharmacodynamics in de novo renal allograft recipients with low (<30%) and high (≥30%) residual expression of NFAT-regulated genes (NFAT-RGE) on tacrolimus treatment

	At d 7			At mo 1			At mo 6		
Laboratory parameter	Low NFAT-RGE N = 40	High NFAT-RGE N = 20	P	Low NFAT-RGE N = 39	High NFAT-RGE N = 20	P	Low NFAT-RGE N = 37	High NFAT-RGE N = 15	P
Tac dose, mg/d	15.8 ± 4.5	13.8 ± 5.5	.124	12.0 ± 4.1	10.7 ± 4.9	.312	6.2 ± 2.9	5.1 ± 2.5	.218
Tac C_0, µg/L	10.1 ± 3.2	8.8 ± 5.0	.204	12.4 ± 4.8	10.0 ± 2.9	.054	9.3 ± 2.6	7.8 ± 2.6	.090
Tac $C_{1.5}$, µg/L	37.5 ± 16.1	21 ± 14.7	<.001	31.9 ± 15.6	15.5 ± 7.9	.001	25.1 ± 10.8	15.1 ± 8.7	.007
Tac C/D	0.69 ± 0.28	0.84 ± 0.89	.347	1.14 ± 0.44	1.19 ± 0.83	.769	1.79 ± 0.88	1.89 ± 0.88	.744
IL-2 RGE, %	7 ± 7	28 ± 22	.001	22 ± 34	40 ± 28	.075	32 ± 26	54 ± 25	.016
IFN-γ RGE, %	13 ± 9	35 ± 22	<.001	24 ± 25	48 ± 24	.003	39 ± 24	61 ± 24	.010
GM-CSF RGE, %	7 ± 6	26 ± 20	<.001	18 ± 26	39 ± 28	.011	31 ± 26	51 ± 25	.029
NFAT-RGE, %	9 ± 7	30 ± 21	<.001	21 ± 28	42 ± 27	<.001	34 ± 25	55 ± 24	.015

C, concentration; C0, trough level; C/D, concentration-dose ratio; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN-γ, interferon-γ; IL-2, interleukin 2; NFAT, nuclear factor of activated T cells; p, significance; RGE, residual gene expression; Tac, tacrolimus

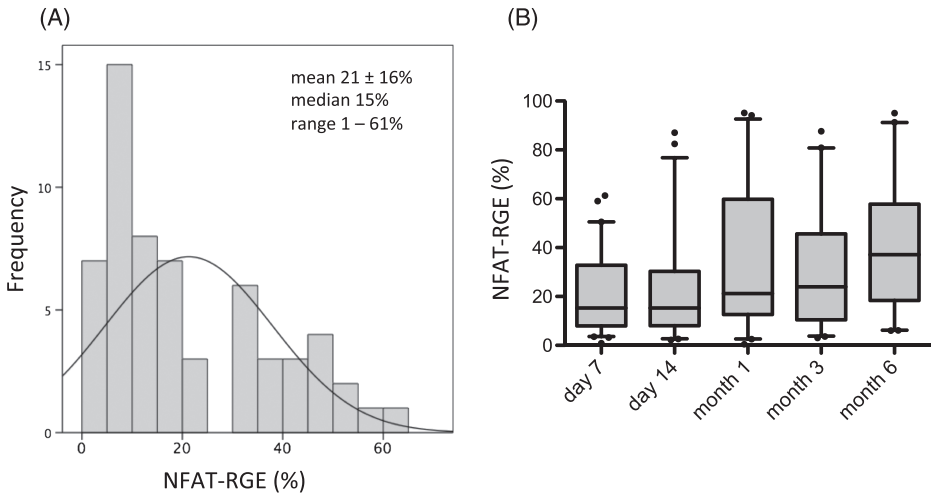

FIGURE 3 (A) Residual nuclear factor of activated T cells-regulated gene expression (NFAT-RGE) in tacrolimus treated renal allograft recipients early after transplantation (day 7), and b) during the 6-month study period (median, 5 and 95% percentiles are shown)

TABLE 3 Adverse events in de novo renal allograft recipients with low (<30%) and high (≥30%) residual NFAT-regulated gene expression (NFAT-RGE)

	Low NFAT-RGE (<30%)	High NFAT-RGE (≥30%)	*P*-value
Combined endpoint	2 (5%)	7 (35%)	.002
Death	0	1 (5%)	.154
Transplant failure	0	1 (5%)	.154
Treated BPAR	2 (5%)	7 (35%)	.002
GFR >60 mL/min	11 (27.5%)	6 (30%)	.694
GFR <30 mL/min	4 (10%)	5 (25%)	.092
Any infection	28 (70%)	9 (45%)	.060
All viral	22 (55%)	5 (25%)	.028
CMV	13 (32.5%)	1 (5%)	.018
BKV	8 (20%)	1 (5%)	.125
CMV and/or BKV	21 (52.5%)	2 (10%)	.001
Bacterial	10 (25%)	5 (25%)	1.00
Fungal	1 (2.5%)	2 (10%)	.209
Malignancy	0	0	-
De novo/worsening hypertension	2 (5%)	2 (10%)	.464
De novo diabetes	2 (5%)	5 (25%)	.023
De novo hyperlipidaemia	1 (2.5%)	2 (10%)	.209
Neurotoxic signs (tremor/trembling)	5 (12.5%)	5 (25%)	.221
Other events	4 (2.4%)	3 (10.5%)	.570

BPAR, biopsy-proven acute rejection; BKV, polyoma (BK) virus; CMV, cytomegalovirus; GFR, glomerular filtration rate; NFAT, nuclear factor of activated T cells; RGE, residual gene expression

NFAT-RGE ≥30% (combined endpoint: 35 *vs.* 5%, *P* = .002; Table 3). There was 1 death and 1 graft failure in patients with low residual expression of NFAT-regulated genes. This 2 patients also showed BPAR.ROC analyses for the ability of NFAT-RGE to discriminate patients at risk for the combined endpoint (death, transplant failure and acute rejection) from stable patients showed a good diagnostic accuracy (*P* = .005; AUC = 0.797; 95% CI 0.607–0.988; Figure 4). Simultaneous maximization of sensitivity and specificity obtained from ROC curves was shown at NFAT-RGE of 30% (sensitivity 77.8%, specificity 80.4%), whereas the positive predictive value was 0.35 and the NPV was 0.95.

BPAR occurred in 9 cases, 7 patients with NFAT-RGE ≥30% and 2 in patients with NFAT-RGE <30% (35 *vs.* 5%, *P* = .002; Figure 5). Histopathological classifications of these 2 patients demonstrated a subclinical or borderline acute rejection.

Patients with a BPAR demonstrated a high residual expression of NFAT-regulated genes early after renal transplantation (35 ± 23 *vs.* 12 ± 13%, *P* < .001; Figure 6A).

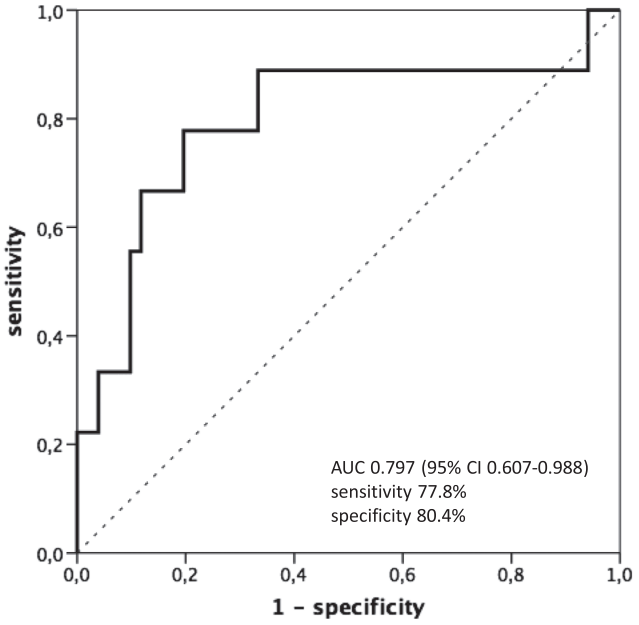

FIGURE 4 Receiver operating curve on nuclear factor of activated T cells-regulated gene expression (threshold 30%) de novo renal allograft recipients at risk for the combined endpoint (death, transplant failure, and acute rejection; area under the curve [AUC] 0.797; 95% confidence interval [CI] 0.607–0.988, *P* = .005)

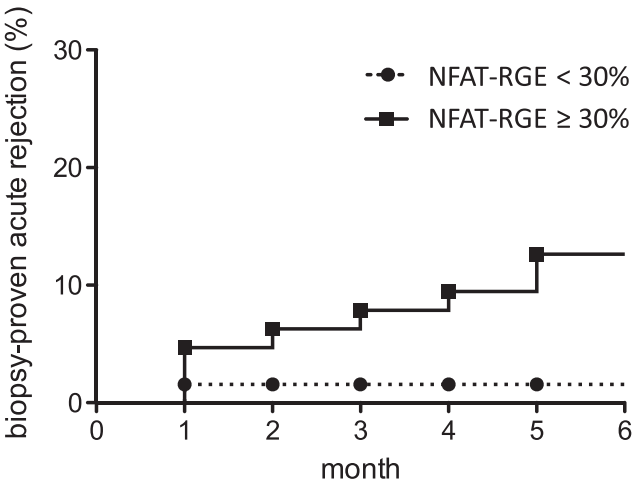

FIGURE 5 Incidence of biopsy-proven acute rejection in renal allograft recipients with low (<30%) and high (≥30%) residual expression of nuclear factor of activated T cells-regulated genes (NFAT-RGE) in the early post-transplant period

3.6 | Safety

Renal allograft function was comparable in both patient cohorts at month 6 with an S-creatinine of 124 ± 53 *vs.* 142 ± 59 μmol/L, *P* = .247 (estimated glomerular filtration rate 56 ± 27 *vs.* 52 ± 18 mL/min, *P* = .525). Proteinuria was comparable at month 6 with 0.167 ± 0.192 *vs.* 0.152 ± 0.106 g/L, *P* = .869.

Infectious complications resulting in a hospitalization occurred in 70% of patients with NFAT-RGE <30% compared to 45% in patients with NFAT-RGE ≥30% (*P* = .060; Table 3). In particular, viral infections were significantly higher in the patient group with NFAT-RGE <30% compared to patients with NFAT-RGE ≥30% (55 *vs.* 25%, *P* = .028; Figure 7). More than half of the patients with NFAT-RGE <30% experienced CMV or BKV replication (52.5 *vs.* 10%, *P* = .001). Incidences of bacterial or fungal complications were comparable in both groups.

Altogether patients with a viral complication, in particular CMV or BKV infection, showed a significantly lower residual expression of NFAT-regulated genes (10 ± 7 *vs.* 19 ± 19%, *P* = .010). In contrast, median values for Tac C_0 and Tac $C_{1.5}$ in both groups were comparable (Figure 6B).

No malignancy occurred in either of patient groups. The incidence of drug-related adverse events (neurotoxic, e.g. trembling, de novo or worsening hypertension, or hyperlipidaemia) were comparable (Table 3). New-onset diabetes occurred more often in the group with NFAT-RGE ≥30%, i.e. with low inhibition of NFAT-RGE, showing the effect of steroids on the development of diabetes in the early post-transplantation period (Table 3).

4 | DISCUSSION

Optimized immunosuppression might be possible by individualized CNI treatment monitored by pharmacodynamic tools that determine the immune response.[6,7] The ultimate goal would be a balance between the optimal suppression of alloresponses while preserving maximal immunocompetence towards viral infections, reactivation, and virus-induced malignancy. Although both responses share the same mechanistic principle, the different level of effector responses allows *fine-tuned* therapeutic intervention. Also, treating patients with a minimal required dose of a CNI might reduce *off-target* side effect. Considering the proposed mode of action of CNIs, the transcriptional activity of NFAT-regulated genes might be a useful immune monitoring tool to individualize CNI therapy.[19]

In the present European multicentre study in renal allograft recipients on Tac treatment, NFAT-RGE was monitored in addition to standard Tac treatment. Patients on Tac treatment showed a high interindividual variability of NFAT-RGE. Patients with low inhibition of IL-2, IFNγ and GM-CSF gene expression resulting in a high NFAT-RGE could be identified; Tac doses and Tac C_0 levels, which represent the standard monitoring tool in transplantation, were comparable in this high NFAT-RGE group to patients with RGE <30%. These latter patients experienced the combined endpoint of BPAR, graft loss, and death with a significantly higher incidence. Acute rejection episodes occurred more often and earlier after transplantation if NFAT-RGE was ≥30% compared to patients with stronger inhibition of NFAT-RGE. An NPV of 0.95 at NFAT 30% led to better identification of patients without rejection risk. By contrast, patients with low NFAT-RGE were at increased risk of infectious complications, especially viral infections. In patients with CMV or BKV infection or

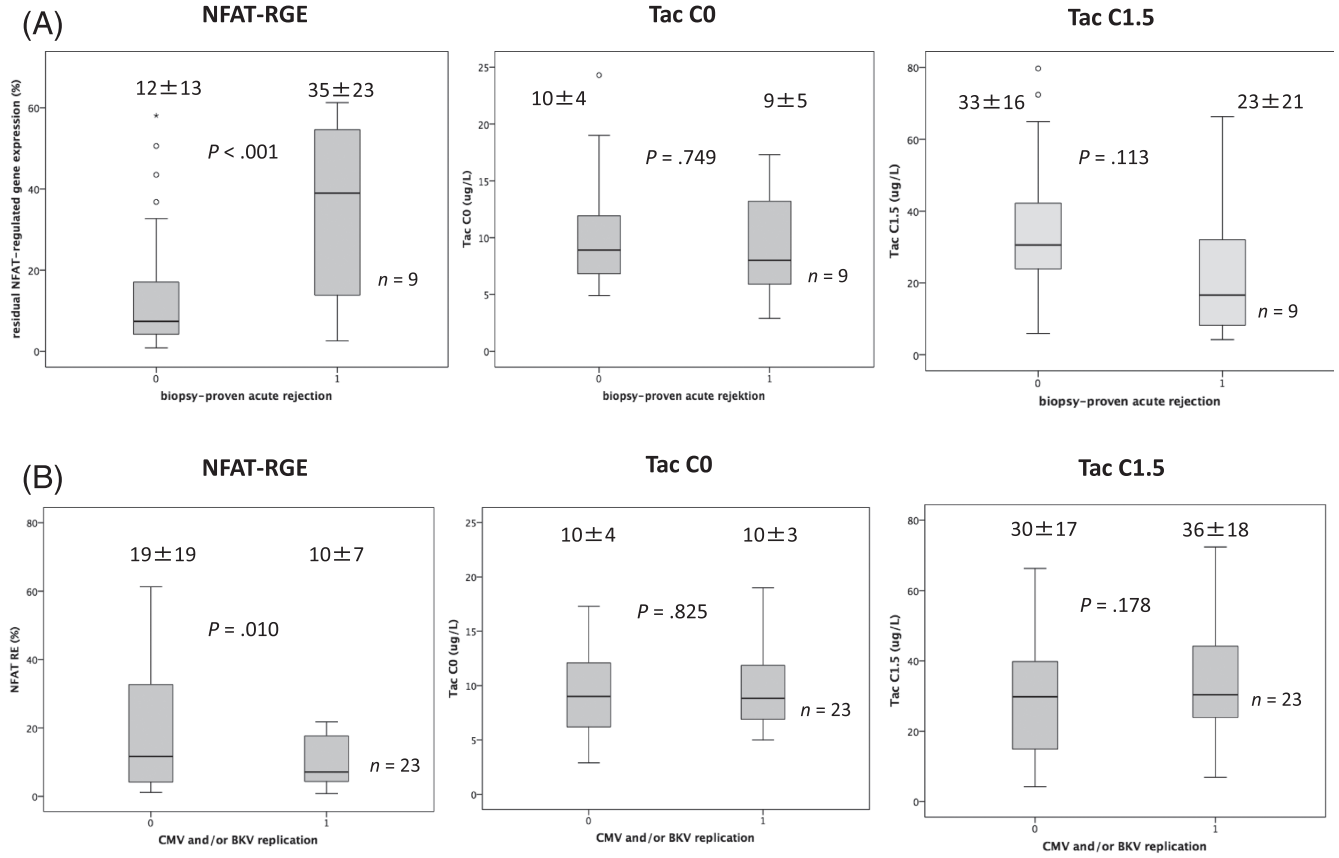

FIGURE 6 Pharmacokinetic and pharmacodynamics results in patients with (1) and without (0) (A) biopsy-proven acute rejection, and (B) cytomegalovirus (CMV) and/or polyoma virus (BKV) infection. NFAT-RGE, nuclear factor of activated T cells-regulated gene expression

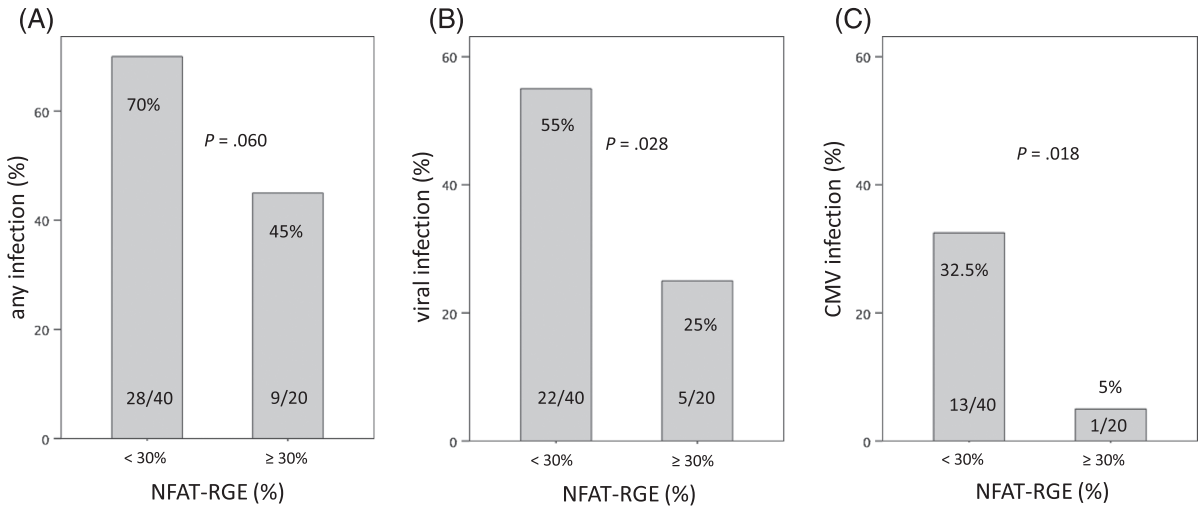

FIGURE 7 Incidence of (A) any infection, (B) viral infection and (C) cytomegalovirus (CMV) infection in renal allograft recipients with low (30%) and high (≥30%) residual expression of nuclear factor of activated T cells-regulated genes (NFAT-RGE)

replication within the first 6 months after transplantation, NFAT-RGE was low (<10%).

The present study represents the first prospective multicentre study on standard Tac treatment monitored by the immune monitoring tool residual NFAT-RGE immune monitoring tool.The RT-PCR technique provides a rapid, highly reproducible and sensitive tool for the quantitative analysis of gene expression.[18] This assay can be semi-automated, standardized and performed in laboratories using reverse transcription–PCR.[34] Although overall gene expression is reduced within 24 hours upon shipment, the relative degree of NFAT

inhibition remains stable during this period. NFAT-RGE has shown low analytical variability in repeated measurements (<10%). Whereas interpatient variability is high, an earlier evaluation demonstrated low intraindividual variability in patients on stable CNI doses and stable immunosuppressive comedication.[21]

In earlier studies, the linearity, precision and limit of quantification, as well as sample stability, were investigated.[18,22,34,35] For interlaboratory comparison, samples were analysed in 2 experienced laboratories. This analysis demonstrated that this assay could be set up with satisfactory analytical performance in a routine molecular biological laboratory with results comparable between laboratories. The reproducibility of the NFAT-RGE assay across laboratories facilitates the implementation of this assay in different centres for pharmacodynamic monitoring of CNI.

While this tool has already been well evaluated in cross-sectional, prospective observational and interventional studies of CsA-treated adult and paediatric patients with renal, liver, heart or lung transplantations, there are only a few studies in Tac treated patients.[22–24,36–39]

The inverse relationship between Tac levels and gene expression has already been demonstrated in an earlier single-centre study.[29] The highest gene inhibition could be detected at the time of Tac peak levels showing the biological effect of Tac on the 3 evaluated NFAT-regulated genes IL-2, IFNγ and GM-CSF. High interindividual variability of NFAT-regulated genes was observed in CsA- and Tac-treated patients. Combined with a low intraindividual variability, this immune assay may be the optimal tool for monitoring of the biological effect of CNIs.

CNI dose adjustments using NFAT-RGE assists in the individualization of CNI treatment.[40] NFAT-RGE monitoring in addition to PK monitoring expands the possibility and improves individualization of CNI therapy.

The feasibility of individualised CNI immunosuppression by pharmacodynamic monitoring was assessed in the prospective randomized Calcineurin-Inhibitor Sparing trial, and the opportunity to reduce cardiovascular risk and other CNI-induced adverse events were evaluated.[41] NFAT-RGE proved efficacy and safety in individualizing CsA treatment as a translational immune monitoring tool that can reduce cardiovascular risk and improve long-term renal allograft function.[42]

This pharmacodynamic analysis supports the identification of patients at risk of side effects and *overimmunosuppression* and might serve as a reliable assay to lower CNI dosing. In a previous observational single-centre study, NFAT-RGE was low in stable renal allograft recipients with CMV infections.[43] In the present prospective multicentre study, these results were also confirmed in the early post-transplant period.

An independent investigator group studied the feasibility to adjust Tac dosing according to the residual activity of NFAT-regulated genes in renal transplantation in a single-centre clinical study.[44] They suggested that quantitative analysis of the NFAT-RGE might serve as a reliable assay to lower Tac dosing in patients who seem to be *oversuppressed*. However, the assay could not identify patients with insufficient immunosuppression.Another small study from Oslo, Norway supported the potential of NFAT-RGE measurements as a pharmacodynamic tool for additional monitoring of Tac therapy for renal allograft recipients, especially in the context of over-immunosuppression and viraemia.[45] Both independent studies confirmed our previous finding that a considerable proportion of Tac-treated patients show a relatively high residual expression of NFAT-regulated genes without clinical evidence of inadequate immunosuppression.[29] Both Bremer *et al.*[45] and Sommerer *et al.*[29] found a small difference between trough and peak drug levels as a technical explanation for this phenomenon. Since the absolute expression of NFAT-regulated genes in these patients is likewise relatively high, additional regulatory pathways of suppressing alloresponses have to be postulated. So far, previously published additional effects of Tac on other activating pathways and T-cell metabolism[46] could not be confirmed in a clinically relevant physiologic setting.

In an earlier noninterventional trial, NFAT-RGE was assessed in the early post-transplant period in renal allograft recipients on CsA treatment.[47] High residual NFAT-RGE was related to acute rejection episodes and low residual expression with infectious complications. Recently, NFAT-RGE was evaluated as an early noninvasive predictive biomarker for the risk of acute rejection and infection in de novo liver transplant recipients on CsA or Tac treatment.[39] A significant increase in NFAT-RGE was observed in patients with T-cell mediated rejection compared to patients without rejection or infection. However, the present study confirmed the previously identified threshold of NFAT-RGE 30% for patients on Tac-based immunosuppression at risk of acute rejection.[29]

In conclusion, the residual expression of the 3 NFAT-regulated genes (IL-2, IFNγ and GM-CSF) measured by RT-PCR in whole blood samples directly show the biological effect of Tac. The present European multicentre study confirmed this predictive immune marker in de novo renal allograft recipients on Tac treatment. While the standard tools for pharmacokinetic monitoring could not detect patients at risk of acute rejection or viral infections, the immune marker NFAT-RGE clearly shows this possibility. This pharmacodynamic monitoring tool could be added to the standard monitoring, especially in patients at risk of adverse events. This promising new pharmacodynamic biomarker provides physicians with useful information to specify individualized adjustments of treatment and prevent serious clinical events.

4.1 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, and are permanently archived in the Concise Guide to PHARMACOLOGY 2019/20 (Alexander *et al*., 2019 a,b).

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COMPETING INTERESTS

There are no competing interests to declare.

CONTRIBUTORS

Research idea and study design: C.S., K.B., M.B.; data acquisition and study performance: C.S., K.B., M.B., O.M., L.G., P.G.; data analysis/statistics: C.S., T.G.; supervision and mentorship: M.Z., S.M. Each author contributed valuable intellectual content during manuscript drafting, accepts personal accountability for the author's contributions, and agrees to ensure that questions on the accuracy or integrity of any portion of the work are appropriately investigated and resolved.

DATA AVAILABILITY STATEMENT

Research data cannot be shared due to restriction by law.

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