Clinical and Molecular Profiling to Develop a Potential Prediction Model for the Response to Alemtuzumab Therapy for Acute Kidney Transplant Rejection

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Alemtuzumab, a monoclonal antibody that depletes CD52-bearing immune cells, is an effective drug for the treatment of severe or glucocorticoid-resistant acute kidney transplant rejection (AR). Patient-specific predictions on treatment response are, however, urgently needed, given the severe side effects of alemtuzumab. This study developed a multidimensional prediction model with the aim of generating clinically useful prognostic scores for the response to alemtuzumab. Clinical and histological characteristics were collected retrospectively from patients who were treated with alemtuzumab for AR. In addition, targeted gene expression profiling of AR biopsy tissues was performed. Least absolute shrinkage and selection operator (LASSO) logistic regression modeling was used to construct the ALEMtuzumab for Acute Rejection (ALEMAR) prognostic score. Response to alemtuzumab was defined as patient and allograft survival and at least once an estimated glomerular filtration rate (eGFR) > 30 mL/min/1.73 m^2 during the first 6 months after treatment. One hundred fifteen patients were included, of which 84 (73%) had a response to alemtuzumab. The ALEMAR-score accurately predicted the chance of response. Gene expression analysis identified 13 differentially expressed genes between responders and nonresponders. The combination of the ALEMAR-score and selected genes resulted in improved predictions of treatment response. The present preliminary prediction model is potentially helpful for the development of stratified alemtuzumab treatment for acute kidney transplant rejection but requires validation.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?

☑ Alemtuzumab is a monoclonal antibody that has been successfully used for the treatment of acute kidney transplant rejection (AR). However, its efficacy is offset by severe and potentially fatal toxicity. There is an unmet need for prediction tools that enable a more personalized approach to alemtuzumab therapy.

WHAT QUESTION DID THIS STUDY ADDRESS?

☑ This study investigated whether clinical and molecular profiling can be used to develop a prediction model for patient-specific predictions of the response to alemtuzumab therapy.

WHAT DOES THIS STUDY ADD TO OUR KNOWLEDGE?

☑ The combination of 10 clinical variables and 3 mRNA variables resulted in an accurate model for patient-specific predictions on the response to alemtuzumab. Molecular profiling showed that mainly B-cell related genes were associated with the response to alemtuzumab.

HOW MIGHT THIS CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE?

☑ The developed preliminary model is a first step toward a clinical tool that allows stratified alemtuzumab treatment for AR. Moreover, molecular profiling increased our understanding of the mechanism of action of alemtuzumab.

Alemtuzumab is a humanized monoclonal antibody against CD52. Treatment with alemtuzumab leads to a fast, profound, and long-lasting depletion of CD52-bearing cells, including T and B lymphocytes, NK cells, and monocytes.[1,2] The drug is approved for the treatment of multiple sclerosis.[3] In transplantation, alemtuzumab is used as induction therapy.[4,5] Alemtuzumab

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is also an effective therapy for severe or glucocorticoid-resistant acute kidney transplant rejection.[6,7] For these indications, treatment with alemtuzumab compared with anti-thymocyte globulin, results in comparable patient and allograft survival but fewer infusion-related side effects.[2,5–7]

Alemtuzumab has, however, severe side effects, including infection, malignancy, and (life-threatening) auto-immunity.[4,8–11] Furthermore, not all acute rejection (AR) episodes respond favorably to alemtuzumab. There is an unmet need to identify patients who will benefit from anti-rejection therapy with alemtuzumab and to differentiate them from those who will not. For the latter, no treatment, a higher alemtuzumab dose or alternative agents (e.g., anti-thymocyte globulin) might be a better alternative. A personalized approach to alemtuzumab treatment may therefore increase its success rate and limit its toxicity.

Here, multidimensional profiling was used to develop a potential prediction model for the response to alemtuzumab in case of severe or glucocorticoid-resistant acute kidney transplant rejection. In addition to clinical profiling, the incremental predictive value of gene expression profiling using NanoString technology was evaluated. The ALEMtuzumab for Acute Rejection (ALEMAR) prognostic score was constructed that can be used for patient-specific prediction of their response to alemtuzumab.

PATIENTS AND METHODS

Study design and patients

Alemtuzumab is the treatment of choice for severe or glucocorticoid-resistant AR in the Erasmus MC since 2012.[6,7] For this retrospective cohort study, kidney transplant recipients who were treated with alemtuzumab for this indication were investigated.[7] Severe AR was defined as Banff grade IIA or higher. Glucocorticoid-resistant AR was defined as the absence of significant improvement in kidney function as judged by the treating physician. Patients were identified by the electronic medication prescription system of the hospital pharmacy. In this system, all alemtuzumab prescriptions within the Erasmus MC are registered. All kidney transplant recipients who had a biopsy-proven AR and were treated with alemtuzumab between January 1, 2012, and January 1, 2018, at the Erasmus MC were identified using this database and included in the study. Patients who received alemtuzumab as induction therapy were excluded.

Treatment

The first-line treatment for AR was methylprednisolone (1,000 mg) intravenously for 3 consecutive days. In case of acute antibody-mediated rejection (aABMR) or mixed-type rejection, patients also received intravenous immunoglobulins (1 g/kg bodyweight for 2 consecutive days (maximum of 80 g per day)). Patients with severe AR did not receive any rejection therapy prior to alemtuzumab. Before alemtuzumab administration, all patients received prednisolone (50 mg intravenously), acetaminophen (1,000 mg orally), and clemastine (4 mg intravenously) to prevent infusion-related side effects. Next, alemtuzumab was administered subcutaneously as a single dose (30 mg) or 2 doses (30 mg) on 2 consecutive days. Further details of the patients and their treatment were published previously.[7]

Ethical approval

The study was approved by the medical ethical review board of the Erasmus MC (#MEC-2018-1430). A waiver of consent was provided for the reasons that leftover material previously used for diagnostics was used and standard clinical data was extracted.

Clinical data and predictor variables selection

Clinical data of patients and their donors (**Table S1**) was extracted from the hospital electronic patient files. Delayed graft function (DGF) was defined as the need for dialysis in the first week after transplantation. DGF events more than 1 month before the diagnosis of AR were not considered. Rejection type was classified according to the Banff 2017 classification after revision of all biopsies by a nephropathologist (author M.C.C.v.G.).[12,13] For this study, the histological classification was reduced to 3 categories: (1) acute T cell-mediated rejection (aTCMR); (2) aABMR; and (3) mixed-type acute rejection. Donor-specific anti-HLA antibodies (DSAs) were measured using the single-antigen bead assay (Immucor Transplant Diagnostics, Stamford, CT) and analyzed with Match It! Antibody software (Immucor). DSA positivity was assigned according to the software.[14,15] Positive DSA were included when present in the time period from transplantation until 6 months after alemtuzumab treatment.

To assess baseline kidney function before AR, the estimated glomerular filtration rate (eGFR; the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) formula) was collected from 3 months before to the moment of alemtuzumab treatment.[16] The maximum baseline eGFR was defined as the highest eGFR within this timeframe.

All clinical characteristics listed in **Table S1** were considered for inclusion in the prediction model based on clinical relevance to the outcome of interest, observations from an earlier publication studying the same cohort of patients, and literature review.[7,17–19]

Treatment response

The outcome of interest was the response to alemtuzumab, which was evaluated at 6 months after treatment. At this time point, patients were classified as "responders" or "nonresponders." Responders were defined as patients who were alive with a functioning allograft and who had an eGFR > 30 mL/min/1.73 m^2 at least once between day 7 and 6 months after alemtuzumab. Nonresponders were patients who experienced allograft loss or had a functioning allograft whose eGFR did not increase > 30 mL/min/1.73 m^2 after alemtuzumab treatment. Allograft loss was defined as the need for dialysis, transplant nephrectomy, or re-transplantation.

Patients who died with a functioning allograft or were lost to follow-up were explicitly not classified as responders or nonresponders, as it was assumed that variables predicting a response with regard to kidney function are distinct from variables that predict death or loss to follow-up. The inclusion of these patients could therefore lead to an unstable and poorly performing prediction model.

Gene expression profiling

To examine the predictive value of gene expression profiling for the response to alemtuzumab, 770 transplantation-related genes

were measured using NanoString technology (Seattle, WA). Detailed methods of the gene expression profiling are provided in **Supplementary File S1**. In brief, formalin-fixed, paraffin-embedded biopsy samples on which the initial diagnosis of AR was made were obtained from the pathology biobank. RNA was isolated as described previously.[20] The Banff-Human Organ Transplant (B-HOT) panel was hybridized to RNA samples at 65°C for 24 hours. The nCounter FLEX system was used for sample preparation, and gene counts were scored by scanning 490 fields-of-view. nSolver software (version 4.0) and the Advanced Analysis module (version 2.0) were used for quality control, normalization, and data analysis.

Statistical analysis

Statistical analysis was performed with nSolver software, SPSS, version 26.0 (IBM, Armonk, NY), and R, version 4.1.0.

Statistical analyses of gene expression data were performed with nSolver and SPSS. To identify the differential expression (DE) genes, statistical models, including negative binomial model and linear regression model were used. The Benjamini-Hochberg (BH) adjustment was used to control the false discovery rate. Statistical analyses on pathway scores and cell type scores were performed in SPSS. Normality of distribution was examined with Kolmogorov-Smirnov testing. Subsequently, either unpaired two-tailed *t*-tests or Mann-Whitney *U* tests were performed as appropriate.

Prediction models were built in R. First, a prediction model with baseline clinical characteristics was constructed with the characteristics listed in **Table S1**. Due to the relatively high number of candidate predictors in relation to the number of patients, we estimated the logistic regression model with a penalized regression by using the least absolute shrinkage and selection operator (LASSO) method to avoid overfitting (LASSO model).[21,22] The nominal variable "type of rejection" consisting of three groups was split into two variables. Other variables were not manipulated and modeled as linear (**Table S1**). One patient had missing data and was excluded from all prediction models for that reason. Two different tuning parameters were tested: the lambda (λ) corresponding to the minimum mean cross-validated error (lamba.min) and the value for which the cross-validated error is one SE of the minimum SE above the minimal λ (lambda.1SE).

Performance of the LASSO models was examined with receiver operating characteristic curves and calibration plots and corresponding Harrell's concordance-index (*c*-index) and slopes, respectively. Internal validations were performed using the bootstrap procedure with 100-times resampling. To correct for overestimation of the model, caused by evaluating the performance of the model on the same data as the development, an optimism corrected *c*-index was calculated using the bootstrap samples. As a sensitivity check for the LASSO model, a standard logistic regression was performed using the strongest predictors from the bootstrap procedure and selecting the appropriate number of variables regarding the event rate. Subsequently, the main model (i.e., LASSO or logistic regression) was chosen based on the highest performance.

Once the main model was defined, the ALEMAR-score was constructed to illustrate the potential clinical use of the model for making patient-specific prognoses for the response to alemtuzumab in AR. To do so, patients were divided in three risk groups: low (< 25%), intermediate (25–40%), and high risk (40–100%) of nonresponse to alemtuzumab treatment. The cutoffs of < 25% and > 40% were chosen, as these were considered as clinically acceptable and nonacceptable risks, respectively. Next, the incremental prognostic value of gene expression analysis was explored by developing a second prediction model (mRNA prediction model) using only patients included in the gene expression analysis (reduced cohort). In this model, the ALEMAR score was used as a predictor variable. Other variables that were considered for inclusion in this model were DE genes that had a log2-fold of change (log2FC) above 1 or below −1 and with a BH-corrected *P* value ≤ 0.1. Correlation matrices were constructed to examine highly correlated DE genes. Based on these correlation matrices and log2FC values, the most promising mRNA genes were selected for inclusion in the prediction model. Again, a LASSO model was developed using a tuning parameter corresponding to the main model (i.e., minimal λ or and 1 SE above minimal λ). The incremental value of the mRNA markers for the prediction of alemtuzumab response was evaluated by calculating the delta (Δ) *c*-index. For the Δ *c*-index, the apparent *c*-index from the main model using only the reduced cohort was subtracted from the apparent *c*-index from the mRNA prediction model.

The Transparent Reporting of a Multivariable Prediction Model for Individual Prognosis or Diagnosis (TRIPOD) guideline (provided in **Supplementary File S1**) was followed for reporting the development of a multivariable prediction model.[23]

RESULTS

Baseline characteristics

Between January 2012 and January 2018, 1,214 patients received a kidney transplant at the Erasmus MC, of which 115 patients (9.5%) were treated with alemtuzumab for AR (characteristics presented in **Table 1**). Sixty-five (57%) of the ARs were diagnosed within the first 3 months after transplantation and aTCMR was the most frequent histological classification (63%). Ninety ARs (78%) were classified as glucocorticoid-resistant, whereas 25 ARs (22%) were classified as severe and required immediate alemtuzumab treatment (i.e., without prior pulse methylprednisolone; **Table 1**).

Treatment outcomes

Treatment outcomes are summarized in **Table 2**. Two patients (1.8%) died with a functioning allograft in the first 6 months after alemtuzumab treatment. One patient was lost to follow-up.

For the remaining 112 patients, the response outcome could be defined at 6 months after alemtuzumab: 84 patients (73%) had a response to alemtuzumab (responders), whereas 28 patients (24%) were nonresponders. Nonresponse resulted from permanent allograft loss in 14 patients (12%) or from not reaching an eGFR > 30 mL/min/1.73 m^2 after treatment in the other 14 patients (12%; **Table 2**).

Development of a prediction model

To predict the response to alemtuzumab at the time of diagnosis of AR, a prediction model was developed with the variables listed

Table 1 Baseline characteristics of the study population (*n* = 115)

Variables	Missing (*N*)[a]	All patients (*n* = 115)	Responders (*n* = 84)	Nonresponders (*n* = 28)	*P* value
Patient characteristics					
Recipient age at transplantation, years, median (IQR)		56.5 (39.4–63.4)	58.7 (43.4–63.6)	48.3 (32.8–57.9)	0.020
Recipient age at AR, years, median (IQR)		56.5 (40.0–63.6)	59.0 (43.5–64.1)	48.4 (33.9–59.1)	0.030
Gender (male), *N* (%)		70 (61%)	52 (62%)	17 (61%)	0.911
Ethnicity, White, *N* (%)		74 (64%)	52 (62%)	19 (68%)	0.375
Primary kidney disease, *N* (%)					0.194
Hypertension		22 (19%)	15 (18%)	5 (18%)	
Diabetic nephropathy		26 (23%)	22 (26%)	4 (14%)	
Glomerulonephritis		9 (8%)	6 (7%)	3 (11%)	
Polycystic kidney disease		20 (17%)	11 (13%)	9 (32%)	
Reflux nephropathy		7 (6%)	7 (8%)	0 (0%)	
Other		28 (24%)	20 (24%)	7 (25%)	
Unknown		3 (3%)	3 (4%)	0 (0%)	
Transplant number (first), *N* (%)		88 (77%)	65 (77%)	21 (75%)	0.849
Pre-emptive transplantation, *N* (%)		40 (35%)	27 (32%)	12 (43%)	0.303
%PRAs – current, median (IQR)		0.0 (0.0–4.0)	0.0 (0.0–4.0)	0.0 (0.0–4.0)	0.428
Transplant characteristics					
Type of donor (living), *N* (%)		80 (70%)	56 (67%)	22 (79%)	0.235
Donor age, years, median (IQR)		54.0 (43.0–63.0)	55.0 (46.0–64.0)	48.5 (39.5–58.0)	0.055
HLA mismatches, median (IQR)	1	4.0 (2.25–5.0)	4.0 (3.0–5.0)	2.5 (2.0–4.0)	0.001
HLA mismatches DR, *N* (%)	1				0.267
0		21 (18%)	13 (16%)	8 (29%)	
1		55 (48%)	40 (48%)	13 (46%)	
2		38 (33%)	30 (36%)	7 (25%)	
Delayed graft function, *N* (%)		33 (29%)	26 (31%)	6 (21%)	0.334
Rejection characteristics					
Timing of rejection (days after transplantation), median (IQR)		18.0 (6.5–348.5)	12.0 (6–134.5)	375.0 (123.3–900.25)	0.001
Early,[b] *N* (%)		65 (57%)	55 (66%)	7 (25%)	0.000
Histological rejection category, *N* (%)					0.012
aTCMR		73 (63%)	53 (63%)	18 (64%)	
aABMR		22 (19%)	20 (24%)	1 (4%)	
MIXED		20 (17%)	11 (13%)	9 (32%)	
DSA, *N* (%)		24 (21%)	17 (20%)	7 (25%)	0.595
Max baseline eGFR,[c] median (IQR)		35 (15.1–50.5)	36.5 (14.8–50.2)	27.0 (15.1–55.0)	0.699
Therapy characteristics					
Triple maintenance therapy (TAC + MMF + PRED), *N* (%)		77 (67%)	63 (75%)	11 (39%)	0.001
Dosage frequency of alemtuzumab (single), *N* (%)		101 (88%)	72 (86%)	26 (93%)	0.322
Indication for alemtuzumab (severe AR), *N* (%)		25 (22%)	21 (25%)	3 (11%)	0.111

aABMR, acute antibody-mediated rejection; AR, acute rejection; aTCMR, acute T cell-mediated rejection; DDSA, donor-specific anti-HLA antibody; eGFR, estimated glomerular filtration rate; IQR, interquartile range; TAC, tacrolimus; MMF, mycophenolate mofetil; PRA, panel reactive antibody; PRED, methylprednisolone.
[a]The patient with missing data was excluded from all prediction models. [b]Early rejection < 3 months after transplantation, late rejection > 3 months after transplantation. [c]Max baseline eGFR, highest eGFR in the 3 months prior to alemtuzumab.

Table 2 Treatment outcomes of the study population (*n* = 115)

Variables	Value
Events	
Death with functioning graft,[a] *n* (%)	2 (1.8%)
Time interval (days),[b] mean ± SD	103 ± 42.4
Allograft loss, *n* (%)	14 (12%)
Time interval, days, median (IQR)	93.0 (93.0–98.8)
Lost to follow-up, *n* (%)	1 (0.9%)
Time interval, days	127
eGFR	
Number of measurements,[c] median (IQR)	27.0 (19.0–37.0)
6 months after alemtuzumab, median (IQR)	34.8 (27.0–42.9)
Response to alemtuzumab	
Responders, *N* (%)	84 (73%)
Nonresponders, *N* (%)	28 (24%)

eGFR, estimated glomerular filtration rate; IQR, interquartile range.
[a]Causes of death: cardiac arrest during pneumonia and cardiac decompensation (day 73 after alemtuzumab); pneumosepsis (day 133 after alemtuzumab). [b]Days after alemtuzumab treatment. [c]Number of eGFR measurements during the follow-up period.

in **Table S1**. The minimal λ parameter in the LASSO procedure shrunk five variables to zero: DGF, donor age, DSA, pre-emptive transplantation, and type of donor (**Figure 1**). Other variables remained in the minimal λ (lambda.min) model (corresponding coefficients are listed in **Table S2**). Recipient age at AR, panel reactive antibody (PRA)-current, number of HLA-mismatches, and maximum baseline eGFR were all positively associated with the alemtuzumab response (i.e., a higher value of the variable resulted in a higher chance of response). Receiving triple maintenance therapy, early timing of rejection, a double dosage of alemtuzumab, type of rejection aABMR, and severe AR (in comparison to glucocorticoid-resistant AR) were all associated with a higher chance of response. Mixed-type AR was related to a lower chance of response (compared with aTCMR and aABMR; **Table S2**).

Performance measures of the lambda.min model showed a good discrimination and calibration as shown in the receiver operating characteristic curve and calibration plot, with a corresponding apparent *c*-index = 0.858 (95% confidence interval (CI) 0.778 to 0.937) and a calibration slope close to 1 (intercept = 0.849, slope = 1.955; **Figure S1**). The LASSO model using a tuning parameter to 1 SE above minimal λ (lambda.1SE model) showed lower performance compared with the lambda.min model and was therefore not included in further analysis (**Figure 1**, **Figure S1**, **Table S3**).

Internal validations were performed for the lambda.min model with a bootstrap procedure. **Table S4** shows how often the different variables were selected in the 100-times remodeling. The internal validation provided an estimate for the optimism-corrected c-index, which resulted in a minor decrease in the discriminating performance (optimism corrected *c*-index: 0.795).

As a sensitivity check for the lambda.min LASSO model, a logistic regression analysis was performed using the strongest coefficients from the bootstrapped results: "HLA-mismatches," "triple maintenance therapy," and "timing of rejection." Timing of rejection showed no significance in the logistic regression model (data not shown) and was therefore replaced by "maximum baseline eGFR." Odds ratios, 95% CIs and corresponding *P* values are presented in **Table S5**.

Discrimination of the logistic regression was lower compared with the lambda.min LASSO model (apparent c-index log model = 0.814; 95% CI 0.712 to 0.916), whereas the calibration slope was slightly closer to one (intercept = 0.000, slope = 1.000; **Figure S1**). As discrimination is superior to calibration as a performance measure in developmental prediction models, the lambda.min LASSO model was selected as the main model.[23]

ALEMAR score for patient-specific prognosis

The main model was used to derive the prognostic ALEMAR score to predict the response to alemtuzumab in acute kidney transplant rejection. Patients were divided in low- (< 25%), intermediate- (25–40%), and high-risk (40–100%) of nonresponse to alemtuzumab therapy. Response occurred in 68 (91%) of low-risk patients, in 11 (69%) of intermediate-risk patients, and in 7 (30%) of high-risk patients (**Table 3**). The equation of the ALEMAR score includes all variables and their corresponding coefficients derived from the main model. By filling in the values of the variables for each patient, the probability of nonresponse to alemtuzumab can be calculated. The equation is provided in **Supplementary File S1**.

Gene expression profiling

To evaluate the additional value of transplantation-related genes for the prediction of alemtuzumab response, the gene expression profile using the B-HOT panel was analyzed. Leftover material of the diagnostic biopsy could be identified for 91 patients, of which 63 samples passed the quality control and normalization procedure in nSolver analysis (**Figure S2**). Baseline characteristics and treatment outcomes of these 63 samples are provided in **Tables S6** and **S7**, respectively.

Unsupervised clustering and DE analysis

Unsupervised clustering of gene expression profiles did not separate responders from nonresponders to alemtuzumab (**Figure 2a**). The DE gene analysis, however, revealed multiple genes that were differentially expressed between responders and nonresponders (**Figure 2b**). Thirteen DE genes met the significance cutoff (log2FC > 1 or < −1 and adjusted *P* value < 0.1; **Table S8**). Twelve genes had higher expression in nonresponders and included nine genes involved in B lymphocyte function, specifically in the production of immunoglobulins (XBP1, PRDM1, IGHM, IGKC, IGLC1, and IGHG1-4) and one gene that encodes for the presence of mast cells within tissue (TPSAB1/B2). One gene was expressed higher in responders (S100A8). The protein encoded by this gene has been related to better outcome of kidney transplants.[24,25]

High B-cell receptor signaling scores in nonresponders

In addition to unsupervised clustering and DE analysis, pathway analysis was performed. The pathway of B-cell receptor signaling (BCR) was significantly higher in nonresponders

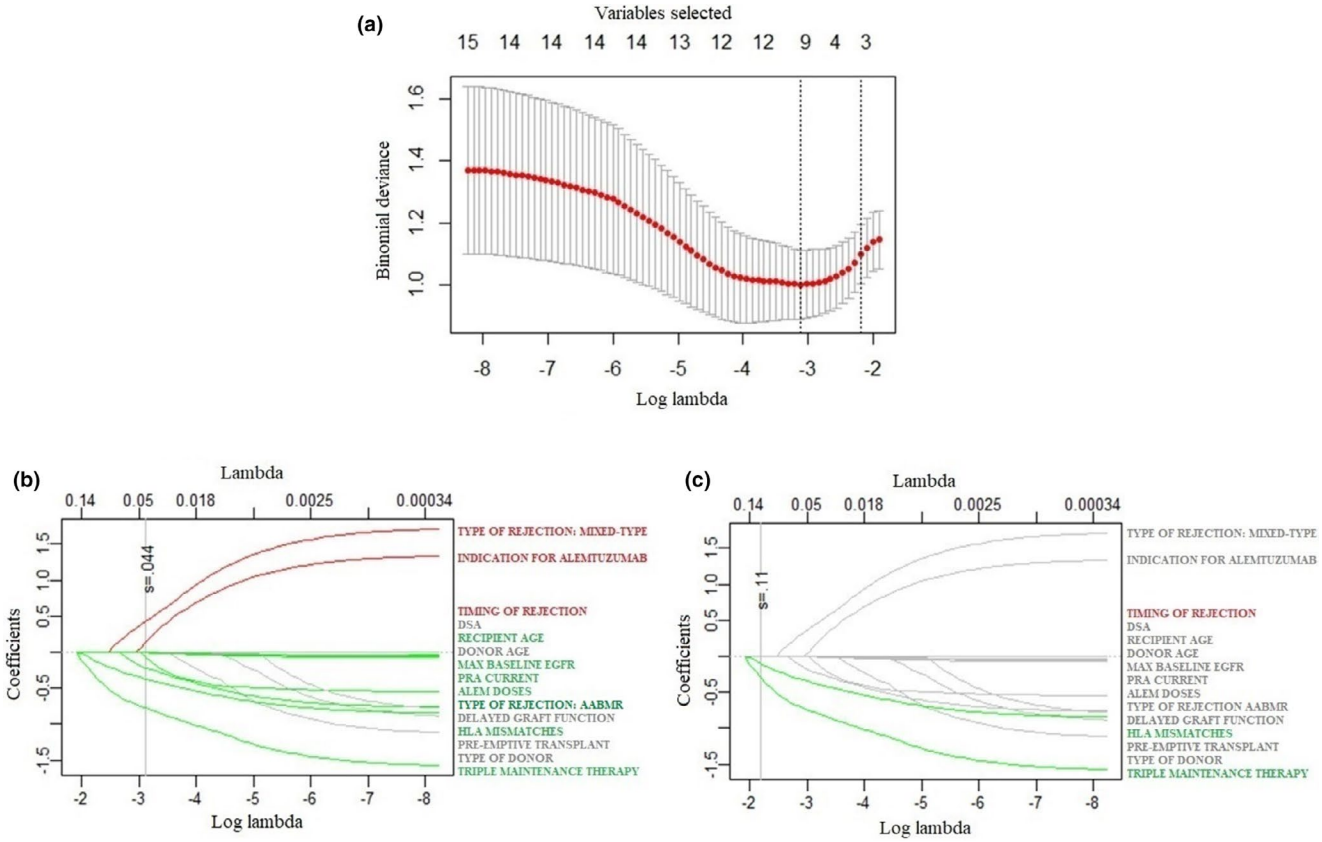

Figure 1 Penalization and shrinkage of predictor variables with LASSO method. LASSO method was used for shrinkage and selection of variables to include in the prediction model for patient specific prognosis on alemtuzumab response. (**a**) Two tuning parameters were tested corresponding to the minimal cross validated error (lambda.min) and to a value of 1 standard error (SE) above the minimum (lambda.1SE), as shown by the left and right dotted vertical lines respectively. (**b**) The shrinkage factor (s = 0.44) corresponding to lambda.min resulted in exclusion of 5 variables (grey), the other 10 variables remained in the model. Positive variables (red) give a higher risk of non-response to alemtuzumab, while negative variables (green) give a lower risk of nonresponse. (**c**) The shrinkage factor (s = 0.11) corresponding to lambda.1SE resulted in inclusion of 3 variables. Other variables were shrunken to zero (grey). LASSO, least absolute shrinkage and selection operator. [Colour figure can be viewed at wileyonlinelibrary.com]

compared with responders (P = 0.007; **Figure 3a**). **Table S9** depicts the genes that are included in this BCR score. Other molecular pathways and cell types that are incorporated in the B-HOT panel, were not different between responders and nonresponders (data not shown).[26]

On visual examination, a vertical separation of samples according to BCR scores was identified in both responders and nonresponders. By clinical data examination, it was found that patients with high expressing BCR were almost exclusively patients who had a relatively late AR (> 3 months after transplantation) both in responders (P < 0.001) and in nonresponders (P = 0.030). Among the patients with a late rejection, nonresponders still had a significantly higher BCR score compared with responders (P = 0.033; **Figure 3b**).

Next, BCR scores in relation to the histological rejection classification were examined as the diagnosis of aABMR or mixed-type AR was considered as a plausible confounder for high BCR scores. However, the diagnosis of aABMR plus mixed-type was not related to the BCR score (P = 0.753; **Figure S3**).

The incremental prognostic value of gene expression profiling

The incremental value of gene expression profiling to predict the response to alemtuzumab was assessed by constructing a second

Table 3 ALEMAR score for patient specific prognosis

Risk group	Probability of nonresponse	Patients (*n*)	Response (*n*)	Response rate
Low	0–25%	75	68	91%
Intermediate	25–40%	16	11	69%
High	40–100%	23	7	30%

ALEMAR, ALEMtuzumab for Acute Rejection.

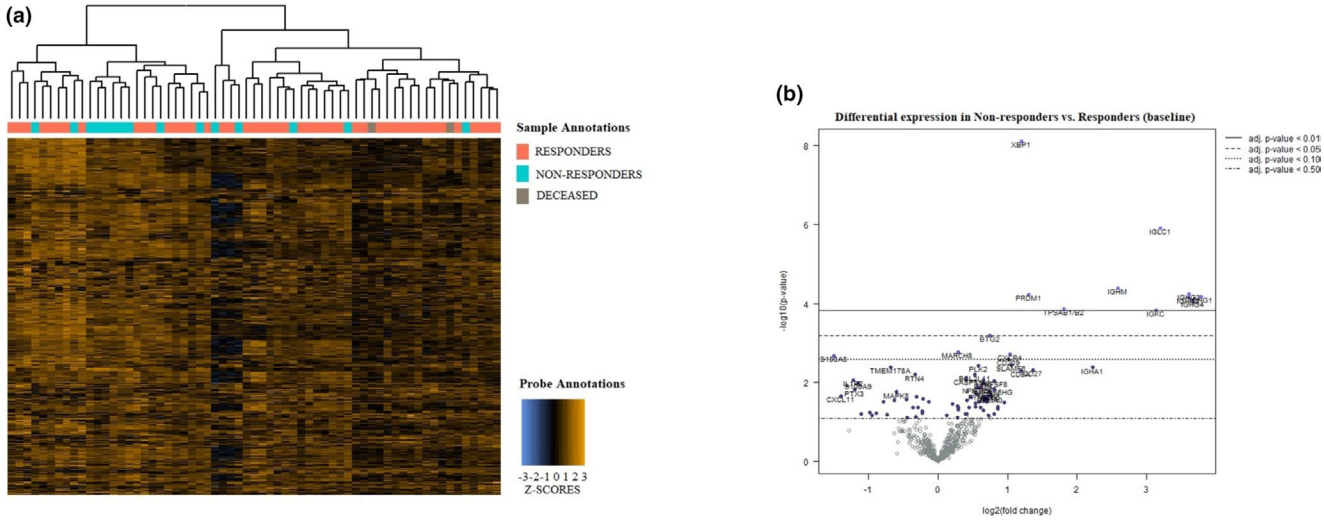

Figure 2 Gene expression profiling using NanoString Technology – Unsupervised clustering and Differential expression of genes. Gene expression profiling using the Banff-Human Organ Transplant (B-HOT) panel of NanoString Technology. (**a**) Unsupervised hierarchical clustering of the normalized data of the 758 genes measured in biopsy samples collected from alemtuzumab-treated patients (n = 63). The unsupervised clustering did not separate responders from nonresponders. (**b**) Volcano plot of differential gene expression (DE) shows multiple genes that were different between responders compared to nonresponders (baseline). The degree of statistical significance according to Benjamini-Hochberg adjusted P values (adj. P value) is indicated with horizontal lines. [Colour figure can be viewed at wileyonlinelibrary.com]

prediction model (mRNA prediction model), including all patients who were included in the gene expression profiling except for one patient with missing clinical data (n = 62). The response rate (n = 46, 73%) in the mRNA model was equal to the nonresponse rate in the main model (n = 83, 73%; **Table 2**, **Table S7**).

Examination of correlation matrices of DE genes (**Figure S4**) and log2FC values (**Table S8**) resulted in the selection of 4 mRNA markers to include in the mRNA prediction model: XBP1, IGHG1, CXCR4, and S100A8. After LASSO shrinkage, all predictor variables were kept in the model (**Figure S5**). Coefficients of the predictor variables are provided in **Table S10**.

The discrimination of the mRNA prediction model was excellent with a c-index of 0.918 (95% CI 0.840 to 0.993; **Figure 4**). The mRNA prediction model showed an incremental predictive value compared with the main model with a Δ c-index of 0.063.

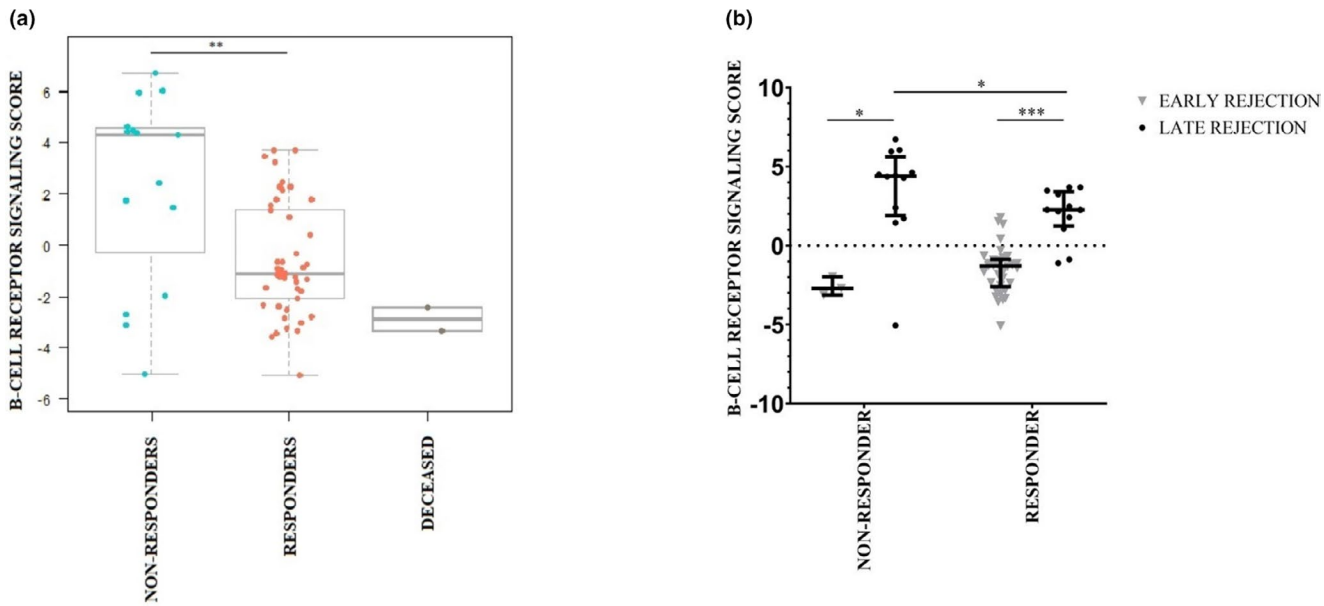

Figure 3 B-cell receptor signaling score. NanoString pathway analysis for B-cell receptor signaling (BCR) related genes calculates a score for each patient based on the overall expression of BCR genes. (**a**) This BCR score was significantly higher in nonresponders compared with responders (P = 0.006). (**b**) A significant association was found between this BCR score and the timing of acute rejection (AR) irrespective of response to alemtuzumab (nonresponders: early vs. late P < 0.001, responders: early vs. late P = 0.030). Among the patients with late rejections, nonresponders had a significantly higher BCR score compared with responders (P = 0.033). [Colour figure can be viewed at wileyonlinelibrary.com]

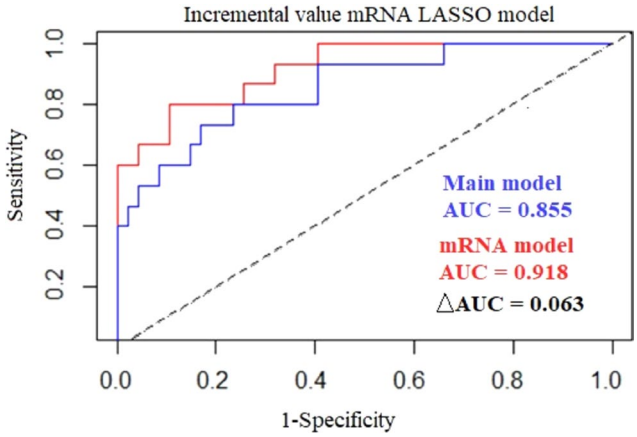

Figure 4 Performance of the mRNA LASSO model. The main model using the reduced cohort had an AUC/c-index of 0.855. The discrimination of the mRNA LASSO model—that includes mRNA markers and the ALEMAR score as predictors—was excellent (AUC/c-index = 0.918). The ΔAUC of the mRNA model is 0.063. *AUC, area under the curve. ALEMAR, ALEMtuzumab for Acute Rejection; LASSO, least absolute shrinkage and selection operator. [Colour figure can be viewed at wileyonlinelibrary.com]

DISCUSSION

In this study, a multidimensional prediction model was developed to make a patient-specific prognosis regarding the response to alemtuzumab in severe and glucocorticoid-resistant acute kidney transplant rejection. In addition, gene expression profiling revealed that B cell-related gene expression was related to the response to alemtuzumab. The incorporation of selected genes in this preliminary prediction model resulted in significant model improvements.

The ALEMAR score included several predictors that were previously associated with a good response to other (non-alemtuzumab) AR treatments, such as an early timing of AR, higher recipient age, and a high baseline eGFR.[27–32] Timing of rejection in particular seems to play an important role in the response to alemtuzumab, as reflected in the hardly overlapping ranges in timing between responders and nonresponders (**Table 1**). It was also found that a double alemtuzumab dose and the treatment indication "severe AR" were related to a higher chance of response. An unexpected finding is that a higher number of HLA mismatches and a high %PRA were correlated with a better response, as, in general, a higher number of HLA-mismatches and PRA are associated with inferior transplant outcomes.[33,34] It could be hypothesized that the HLA-mismatch and %PRA are not causally related to the response but reflect confounding of other unknown variables that were not included in the present model. Exploration of the potential incremental value of incorporation of gene expression profiling in the prediction model showed that the model performance increased substantially. Previous studies either investigated the use of molecular profiling as a single modality for treatment predictions or only used clinical characteristics.[19,25,35] The combination of molecular profiling with clinical and histological characteristics in the development of a prediction model for AR treatment response is novel and resulted in a strong predictive model. Validation and confirmation in a larger and external cohort of patients should be performed to confirm the incremental predictive value of gene expression profiling and the predictive power of the model. Although the incremental value of gene expression profiling in the model was significant, it can be hypothesized that the main model is already sufficient for patients with a low or high-risk score prognosis. For patients with an intermediate risk prognosis, the main model might not be sufficient to decide whether or not to treat with alemtuzumab. Especially for these patients, it may be worthwhile to perform additional gene expression measurements and generate a more precise prognosis.

In addition to its predictive value for patient-specific prognosis, gene expression profiling provided an interesting finding with regard to the pathophysiology of AR, as it was revealed that the majority of upregulated genes in nonresponders are related to B lymphocyte function. This is in line with previous studies that reported an association between the presence of B lymphocytes and poor graft outcomes in patients with AR.[36–38] In addition, the BCR score was also related to the response of alemtuzumab. We ruled out that these BCR score differences were caused by the number of aABMR or mixed-type rejections. This suggests that a histological classification of aTCMR does not exclude a role of B lymphocytes in its pathophysiology. Previous studies demonstrated B lymphocyte infiltrates in aTCMR biopsies.[38–40] In addition, it was found that BCR scores were especially high in patients with late AR, suggesting that late rejections differ in pathophysiology from early rejections and may require different treatment. A relation between the presence of B lymphocytes in biopsies and late-onset AR was described previously.[39]

The association of B cell genes with alemtuzumab response is interesting from the perspective of alemtuzumab pharmacokinetics and dynamics. First, the expression of CD52 differs between subsets of lymphocytes.[41] Of special interest, is the finding that less than 50% of plasma cells in healthy individuals showed CD52 expression.[42] Therefore, it might be that high B cell gene expression levels represent a specific B cell subset—for example, plasma cells—that express CD52 not at all or only in low amounts, and is therefore not depleted by alemtuzumab. In this regard, it could be hypothesized that patients with a high B cell gene signature might be more effectively treated with a specific plasma cell-depleting drug, such as daratumumab.[43] Second, alemtuzumab may not reach the same concentration in tissues as compared with peripheral blood and thus might lead to incomplete depletion of tissue-resident lymphocytes. No reports have been published on the distribution of alemtuzumab into kidneys and limited data exist on plasma alemtuzumab concentrations following a fixed dose.[44,45] Several human and mice studies reported incomplete depletion of lymphocytes from lymph nodes, spleen, and skin after alemtuzumab treatment, despite complete depletion of lymphocytes from peripheral blood.[46–49]

The present study is limited by the relatively small patient numbers and the low number of nonresponse events. Although the LASSO method guards against overfitting, an external validation is required to obtain more certainty concerning the models. The present study should therefore be interpreted as a first step for the development of a prediction model rather than a definite model. It should also be noted that the developed models predict

the response in terms of kidney function but do not give any information about the toxicity of alemtuzumab. Ideally, a prediction model should also provide clinicians with a patient-specific prognosis regarding side effects, including viral infections, such as BK viremia, which are a well-known complication of alemtuzumab treatment.[7] Another limitation is that the heterogeneity of the patient cohort made it difficult to define treatment response. The eGFR cutoff we used has been used previously.[50] Despite not being ideal, this definition was considered as the most optimal for the study purpose The criterion "at least once" and the relatively early time point at 6 months, were chosen to guard against nonrelated adverse events that influence kidney function that would possibly lead to an incorrect conclusion of nonresponse to alemtuzumab. The same holds through for the chosen prognostic score cutoffs.

In conclusion, a preliminary prediction model was developed that may be helpful in making a patient-specific prognosis concerning the response to alemtuzumab therapy in acute kidney transplant rejection. Future research should evaluate the generalizability of the model in a larger and external cohort of patients. Moreover, gene expression profiling revealed novel insights into the pathophysiology of acute transplant rejection and raises important questions on the pharmacology of alemtuzumab in need of further investigation. The present findings allow the development of stratified alemtuzumab treatment in acute kidney transplant rejection.

SUPPORTING INFORMATION

Supplementary information accompanies this paper on the *Clinical Pharmacology & Therapeutics* website (www.cpt-journal.com).

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CONFLICT OF INTEREST

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AUTHOR CONTRIBUTIONS

D.M.H.P. and D.A.H. wrote the manuscript. M.C.C.v.G., C.C.B., M.E.J.R., and D.A.H. designed the research. D.M.H.P., M.v.d.Z., M.C.C.v.G., and D.A.M.M. performed the research. D.M.H.P., D.A.M.M., and S.J.B. analyzed the data. S.J.B. contributed new analytical tools.

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