Evaluation of non-invasive biomarkers of kidney allograft rejection in a prospective multicenter unselected cohort study (EU-TRAIN)

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Non-invasive biomarkers are promising tools for improving kidney allograft rejection monitoring, but their clinical adoption requires more evidence in specifically designed studies. To address this unmet need, we designed the EU-TRAIN study, a large prospective multicentric unselected cohort funded by the European Commission. Here, we included consecutive adult patients who received a kidney allograft in nine European transplant centers between November 2018 and June 2020. We prospectively assessed gene expression levels of 19 blood messenger RNAs, four antibodies targeting non–human leukocyte antigen (HLA) endothelial antigens, together with circulating anti-HLA donor-specific antibodies (DSA). The primary outcome was allograft rejection (antibody-mediated, T cell–mediated, or mixed) in the first year post-transplantation. Overall, 412 patients were included, with 812 biopsies paired with a blood sample. *CD4* gene expression was significantly associated with rejection, while circulating anti-HLA DSA had a significant association with allograft rejection and a strong association with antibody-mediated rejection. All other tested biomarkers, including *AKR1C3, CD3E, CD40, CD8A, CD9, CTLA4, ENTPD1, FOXP3, GZMB, ID3, IL7R, MS4A1, MZB1, POU2AF1, POU2F1, TCL1A, TLR4*, and *TRIB1*, as well as antibodies against angiotensin II type 1 receptor, endothelin 1 type A receptor, C3a and C5a receptors, did not show significant associations with

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allograft rejection. The blood messenger RNAs and non-HLA antibodies did not show an additional value beyond standard of care monitoring parameters and circulating anti-HLA DSA to predict allograft rejection in the first year post-transplantation. Thus, our results open avenues for specifically designed studies to demonstrate the clinical relevance and implementation of other candidate non-invasive biomarkers in kidney transplantation practice.

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Lay Summary

Optimal monitoring of kidney allograft rejection is essential to improve patient care but still relies on nonspecific biomarkers (e.g., serum creatinine and proteinuria), circulating anti–human leukocyte antigen (HLA) donor-specific antibodies (DSA) and surveillance biopsies. Blood gene expression profiling and non-HLA antibodies targeting endothelial antigens have shown associations with allograft rejection but remain to be validated in large, prospective, multicentric, and unselected cohorts. We designed the European TRAnsplantation and INnovation (EU-TRAIN) study (ClinicalTrials.gov, NCT03652402) to assess 19 blood mRNAs and 4 non-HLA antibodies in consecutive deeply phenotyped adult patients who received a kidney allograft between November 2018 and June 2020 in 9 European transplant institutions. All patients were prospectively followed from the time of transplantation until 1 year after transplantation. The EU-TRAIN blood biomarkers did not show an additional value beyond standard of care monitoring parameters and circulating anti-HLA DSA to predict allograft rejection. Hence, our results question their clinical utility for monitoring kidney allograft rejection in the first year after transplantation.

Rejection remains an important cause of kidney allograft loss, adversely impacting patient outcomes and constituting a significant public health problem.[1,2] A precise, noninvasive rejection monitoring system is essential to obtain an accurate and timely detection and to provide optimal patient care.[3] However, the field of kidney transplantation lacks robust clinically validated tools for immune monitoring, which still mainly relies on nonspecific biomarkers (e.g., serum creatinine and proteinuria), circulating anti–human leukocyte antigen (HLA) donor-specific antibodies (DSA), and surveillance biopsies.[4] This represents a major issue for routine care, clinical trials, and improvement of transplant outcomes.[4]

Over the past 2 decades, several emerging noninvasive biomarkers have been developed to detect kidney allograft rejection, providing hope to improve the accuracy and timeliness of rejection diagnosis while reducing the need for invasive and costly biopsies.[5–12] Among these, blood gene expression profiling and antibodies targeting non-HLA endothelial antigens, also called non-HLA antibodies, have shown promising performances to detect clinical and subclinical rejection, either antibody-mediated (AMR) or T cell–mediated (TCMR).[7,13,14] Mechanistically driven gene expression levels of blood mRNAs have been associated with spontaneous tolerance and rejection.[6,7,15–19] Most of these genes have been associated with immune regulation, including *FOXP3*, *CTLA4*, and *CD40*, and are expressed by circulating cells, including T, B, and natural killer cells, whose levels have been found altered in rejection events.[20] *TRIB1* and *TLR4* have been described as associated with chronic humoral rejection in case-control studies.[15] Recently, 2 genes, *TCL1A* and *AKR1C3*, were associated and validated as biomarkers of subclinical rejection at 1 year after transplantation in 2 large cohorts.[7] We also hypothesized that tolerance-regulated genes may be modulated in case of immune tolerance breakdown, leading to rejection.[6,7,16–19] Moreover, the association of non-HLA antibodies with auto- and alloimmunity and distinct allograft rejection patterns have also been demonstrated across multiple organ transplants.[21–24]

However, their clinical adoption requires more evidence in specifically designed studies to correctly evaluate the added value beyond standard of care parameters, their context of use, and their transportability.[25–29] There is a need for a robust assessment of these emerging blood biomarkers in a large, deeply phenotyped and unselected cohort of kidney transplant recipients to demonstrate their utility to inform clinical decision-making in routine practice.

To address this issue, we designed the European TRAnsplantation and INnovation (EU-TRAIN) study (ClinicalTrials.gov, NCT03652402), a large prospective longitudinal multicenter cohort study funded by the European Commission (grant agreement no. 754995), to investigate the clinical utility of 23 data-driven and mechanistically informed blood biomarkers (19 mRNA gene expression levels and 4 non-HLA antibodies) in monitoring kidney allograft rejection compared with the standard of care parameters. This study integrates and centralizes on a large scale a protocol-based assessment of clinical, biological, immunologic, and histologic variables using innovative technologies to constitute a multimodal cohort of unselected, extensively phenotyped adult kidney transplant recipients.

In this study, we aimed to (i) investigate the association of blood gene expression profiling and non-HLA antibodies, together with circulating anti-HLA DSA with kidney allograft rejection, and (ii) determine their additional predictive value as compared with parameters collected during the standard of care during the first year after transplantation.

METHODS

Ethics statement

The protocol of this study (ClinicalTrials.gov, NCT03652402) was approved by the institutional review board *Comité de Protection des Personnes du Sud-Ouest et Outre-Mer IV*

(registration approval number: 2018-A00733-52). All patients provided written informed consent to participate during the inclusion visit (i.e., at the time of transplantation). This research is governed by the Commission Nationale de l'informatique et des Libertés (French Data Protection Agency) "Reference Methodology for processing personal data used with the scope of health research" (amended MR-001). The study was conducted in accordance with the Declaration of Helsinki and the International Conference on Harmonization Guidelines for Good Clinical Practice, and the Declaration of Istanbul on Organ Trafficking and Transplant Tourism.

Study design and participants

The EU-TRAIN study is a large, unselected prospective multicenter cohort of deeply phenotyped adult kidney transplant recipients enrolled at the time of transplantation between November 2018 and June 2020 and followed during the first year after transplantation. This cohort is part of a collaborative network involving 9 European transplant centers (Paris-Saint-Louis, Paris-Necker, Nantes, Barcelona-Bellvitge, Barcelona-Vall d'Hebron, Berlin-Charité Mitte, Berlin-Charité Virchow, Geneva, and Paris-Kremlin-Bicêtre), 3 analytical platforms (Paris Cardiovascular Research Center INSERM U970, Center for Research in Transplantation and Translational Immunology UMR 1064 at Nantes University Hospital, and Medical Biology Department of Saint-Louis Hospital), and 1 industrial partner (CellTrend, Luckenwalde, Germany). Detailed inclusion and exclusion criteria are provided in the Supplementary Methods.

Patient recruitment started on the day of the kidney transplantation (baseline visit). Follow-up visits were performed per protocol at 3 months (M3) and 12 months (M12), and additional visits were conducted when a biopsy was clinically indicated (deterioration of kidney function and detection or rise in proteinuria).

We followed the STROBE (Strengthening the Reporting of Observational Studies in Epidemiology) statement checklist for the report of observational cohort studies.[30] We adhered to the STARD (Standards for Reporting of Diagnostic Accuracy Studies) checklist to ensure complete and transparent reporting of study our methods and results for assessing diagnostic accuracy.[31] Moreover, we adhered to the SAGER (Sex and Gender Equity Research) guidelines for reporting sex and gender.[32] Sex was self-reported by participants. All these complete checklists are provided in the Supplementary Material. The overall study design is presented in Figure 1. Details regarding sample size calculation are provided in the Supplementary Methods.

Multidimensional data collection and procedures

Clinical data and laboratory measurements. At baseline visit, clinical and biological data were collected related to (i) recipient characteristics, (ii) donor characteristics, (iii) transplant procedure, (iv) clinical examination, and (v) blood laboratory tests. At follow-up visits (M3 and M12, clinically indicated), a standardized transplant assessment was performed, comprising (i) clinical examination, (ii) immunosuppressive treatment, (iii) blood and urinary analyses for standard of care laboratory parameters, (iv) blood analyses for innovative biomarkers, and (v) kidney allograft biopsy for histologic analysis. To note, allograft instability was defined as a 20% increase in serum creatinine and/or a worsening or appearance of a urine protein-to-creatinine ratio $\geq$0.5 g/g at the time of the follow-up visit.

The complete list of all routine parameters assessed during the study is available in Supplementary Table S1. The detailed immunosuppressive protocol, as well as procedure for data management, quality control, and privacy protection, is provided in the Supplementary Methods.

Immunological phenotyping. Kidney transplant recipients were tested for the presence of circulating donor-specific anti-HLA-A, -B, -Cw, -DR, -DQB, and -DP antibodies at baseline and each follow-up visit with single antigen flow bead assays (One Lambda, Inc.). Beads with a normalized mean fluorescence intensity (MFI) of greater than 500 units were considered positive, and beads with a normalized MFI of greater than 1400 were considered clinically meaningful, as recommended by international guidelines.[33] Immunodominant DSA was defined as the DSA with the highest MFI. All the sera were centrally reviewed in the HLA Saint Louis Hospital Laboratory.

HLA typing of recipients and donors were respectively performed by DNA typing (Innolipa HLA typing kit; Innogenetics) or next-generation sequencing (NGS-go; GenDx) with medium- to high-resolution sequence-specific primer (Linkage Biosciences, One Lambda) according to local HLA laboratory practices.

Histologic and immunohistochemical phenotyping. Kidney allograft biopsies were routinely performed at each follow-up visit according to local centers' practice. One kidney core was paraffin-embedded and formalin-fixed for histologic analysis. C4d staining was performed by immunohistochemistry on paraffin-embedded tissue or by immunofluorescence on frozen tissue according to local practices. Biopsies were assessed by local pathologists blinded to biomarker data, according to the international and standardized Banff 2019 classification for kidney allograft rejection (detailed international Banff scoring system is provided in Supplementary Table S2).[3,34]

Blood biomarkers. We measured 23 biomarkers (19 blood mRNA gene expression levels and 4 non-HLA antibodies) previously studied as potential biomarkers for monitoring kidney allograft rejection,[6,7,13,22] using blood samples collected and processed at each follow-up. All analytic platforms were blinded to the outcome measure (allograft rejection).

We quantified 19 mechanistically driven blood mRNA gene expression biomarkers that were previously associated with spontaneous operational tolerance or rejection (*AKR1C3*, *CD3E*, *CD4*, *CD40*, *CD8A*, *CD9, CTLA4*, *ENTPD1*, *FOXP3*, *GZMB*, *ID3*, *IL7R*, *MS4A1*, *MZB1*, *POU2AF1*, *POU2F1*, *TCL1A*, *TLR4*, and *TRIB1*)[6] using NanoString PlexSet Technology (NanoString Technologies). As previously

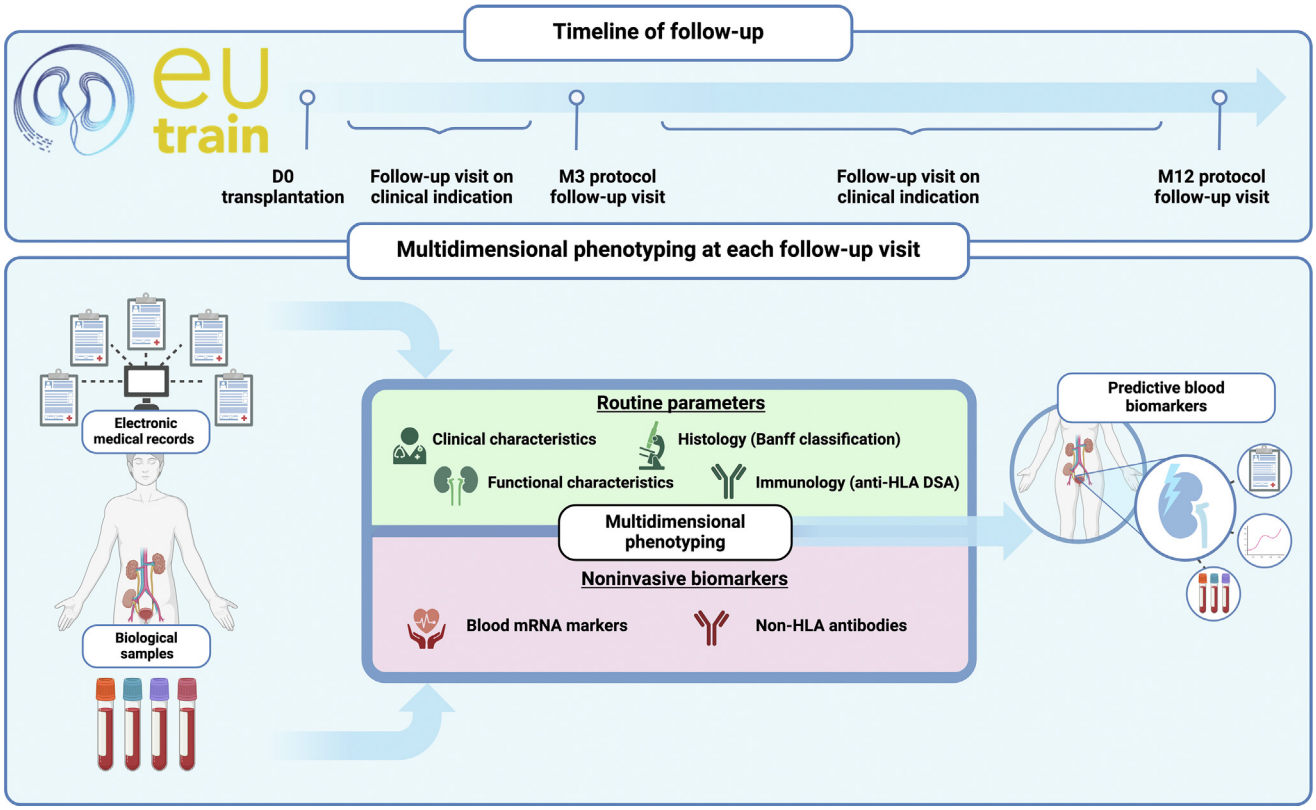

Figure 1 | European TRAnsplantation and INnovation (EU-TRAIN) multimodal biomarker study design. This figure illustrates the recruitment and follow-up of kidney transplant recipients included in the EU-TRAIN study. At each follow-up visit, we performed multidimensional phenotyping, comprising standard of care assessment and noninvasive biomarker evaluation. anti-HLA DSA, anti–human leukocyte antigen donor-specific antibody; ECD, expanded criteria donor. Parts of the figure created with BioRender.com.

described, all normalized counts of blood mRNAs were log2 transformed for subsequent statistical analyses.[7]

We analyzed 4 non-HLA antibodies directed against endothelial targets and involved in auto- and alloimmunity (angiotensin II type 1 receptor [AT1R] antibodies, endothelin 1 receptor type A [ETAR] antibodies, complement C3a receptor [C3aR] antibodies, and complement C5a receptor [C5aR] antibodies) in purified IgG in cooperation with CellTrend with commercially available solid phase assays (One Lambda, Inc.). As previously described in the literature, we measured the antibody levels in U/ml.[22] We used 2 different clinically meaningful positive thresholds for aAT1R antibodies (10 U/ml and 17 U/ml) and 1 positive threshold for ETAR antibodies (10 U/ml), which were associated with allograft rejection in prior studies.[21]

The detailed analytical and data processing procedures are provided in the Supplementary Methods.

Outcomes measures

The primary outcome measure of the study was kidney allograft rejection, assessed blindly to biomarker data at each follow-up visit and defined as either active AMR, chronic active AMR, acute TCMR, chronic active TCMR, or mixed rejection (AMR and TCMR) according to the international Banff 2019 classification.[3] Following the Banff 2019 criteria, borderline/suspicious for acute TCMR cases and equivocal/suspicious for diagnosis of AMR cases were not categorized as rejection.[3]

The secondary outcomes were subtypes of rejection, that is, AMR (referred to as active AMR or chronic active AMR) and TCMR (referred to as acute TCMR or chronic active TCMR).

Statistical analyses

Sample size calculation. Assuming a targeted prevalence of the primary outcome (kidney allograft rejection) of 8%–10% in our population, 0.05 acceptable difference in apparent and adjusted R-squared, and 0.05 margin of error in estimation of intercept, we calculated that the minimal sample size required for model development was approximately 400 patients in our study.[35] Hence, we aimed to assess the eligibility of 550 kidney transplant recipients, considering a potential loss to follow-up of around 25%–30%.

Data transformation and standardization. The distribution of each continuous variable was systematically assessed before analyses. Normality assumptions were evaluated using graphical methods (histogram plots and quantile-quantile plots). In cases where variables exhibited non-normal distributions (e.g., urine protein-to-creatinine ratio), we applied appropriate transformations guided by the nature of data

(e.g., natural logarithm transformation for urine protein-to-creatinine ratio).

Descriptive analyses of baseline characteristics. We described continuous variables using means and standard deviations or medians and interquartile ranges (IQR) if appropriate. Means, medians, and proportions were compared using the Student *t* test (or the Wilcoxon test if appropriate) or the χ^2 test (or the Fisher exact test if appropriate), respectively.

Univariate association of blood biomarkers with kidney allograft rejection and collinearity. As the primary outcome of the study is allograft rejection, defined using kidney biopsies and laboratory measurements, it was predefined in the protocol of the study that the main statistical analysis will include patients who had at least 1 kidney biopsy with a concomitant biology during the study period. Each biopsy was considered as an independent observation.

We assessed the univariate association of the candidate blood biomarkers at the time of biopsy with the primary outcome (odds ratio [OR]) using logistic regression. We measured their discrimination ability through the area under the receiver operator characteristic curve (ROC-AUC). Then, we defined thresholds, according to the literature for AT1R and ETAR antibodies[21,24] and to the maximum of the Youden index (i.e., the optimal cutoff that balances sensitivity and specificity in the best possible way) for other biomarkers without previously published thresholds, to calculate their other individual diagnostic performances to detect kidney allograft rejection (sensitivity, specificity, positive predictive value, and negative predictive value).

Finally, we assessed the collinearity between the biomarkers using a correlation matrix, with statistical correlations calculated using the Spearman test.

Additional value of biomarkers for predicting allograft rejection. We investigated the additional value of the candidate blood biomarkers to predict the individual risk of allograft rejection, as compared with standard of care parameters. For this purpose, we developed and compared elastic net–based predictive diagnostic models of rejection using either solely routinely collected parameters (referred to as the standard of care model), solely blood biomarkers (referred to as the biomarker model), or by integrating routinely collected parameters and blood biomarkers (referred to as the integrative model) at the time of biopsy. We used elastic net regularized regressions because this type of regression performs a feature selection, which allows to get the optimal number of variables in the final model, based on their predictive performances and considering their potential collinearity.

The dataset was randomly divided into train (65%) and test (35%) sets to respectively develop and internally validate the models. To avoid overfitting, combinations of hyperparameters that control the strength and type of applied regularization (alpha "mixing parameter" and lambda "regularization parameter") were optimized by robust 10-fold cross-validation during tuning the models.[36] In addition, the cross-validation process was repeated 5 times to minimize sampling bias. This type of robust internal validation in a targeted validation set that is representative of the intended population and setting may be sufficient to accurately validate the models and demonstrate their applicability in the population of interest.[37]

We used the TRIPOD (Transparent Reporting of a multivariable Prediction Model for Individual Prognosis Or Diagnosis) statement for the reporting of the development and validation of the models. The performances of all predictive models were evaluated based on discrimination through calculation of the ROC-AUC. ROC-AUC were compared using the DeLong's test.[38] The dataset preparation and preprocessing steps, as well as the development and validation of the models, were performed with the *caret* package in R.[39,40]

Handling of missing data. Univariate analyses were performed on complete cases datasets. For multivariable analyses, we performed a single imputation of missing values (if <30%, as recommended by international guidelines[41]) with a random forest algorithm using the *missForest* package in R.[42] The maximum iteration was set to 10 times. Random forest algorithms handle mixed types of missing data, are adaptive to interactions and nonlinearity, and were found to consistently produce the lowest imputation error compared with other imputation methods when data are missing completely at random, as in the EU-TRAIN study due to the systematic data collection.[43,44]

Software. All statistical analyses were carried out using R, version 4.2.2 (R Foundation for Statistical Computing) and Stata software, version 17.0 (StataCorp). *P* values below 0.05 were considered as significant. All the tests were 2 tailed.

Role of the funding sources

The funders of this study had no role in the study design, data collection, analysis, or interpretation of the manuscript.

RESULTS

Study population characteristics

Between November 2018 and June 2020, 412 kidney transplant recipients with at least 1 transplant biopsy with a concomitant biology were prospectively included in the main analysis of the study and followed during a median time of 12.12 months (IQR, 10.79–12.91 months) after transplantation (Figure 2).

The main characteristics of the patients at the time of transplantation are provided in Table 1. The mean recipient age was 53.4 (±14.4) years, 264 (64.1%) patients were male, and 325 (80.6%) patients received transplants from deceased donors. The mean donor age was 55.0 (±15.1) years. A total of 54 patients (13.4%) were retransplanted, and 82 (20.6%) had positive circulating anti-HLA DSA at the time of transplantation. Induction therapy predominantly consisted of rabbit antithymocyte globulin (58.3%).

At the time of biopsy, the mean estimated glomerular filtration rate was 48.4 (±20.1) ml/min per 1.73 m^2, median urinary protein-to-creatinine ratio was 0.2 g/g (IQR, 0.1–0.4

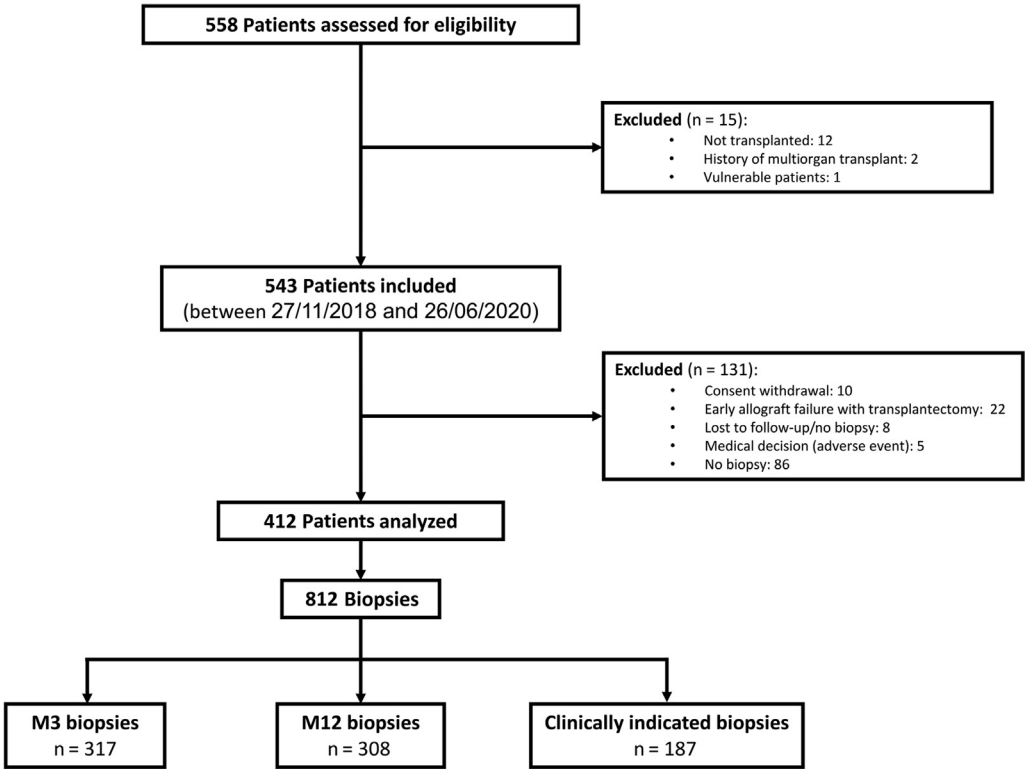

Figure 2 | Flowchart of the European TRAnsplantation and INnovation (EU-TRAIN) study. M3, 3 months; M12, 12 months.

g/g), and positive circulating anti-HLA DSA were detected in 151 (19.6%) cases. The most frequently used immunosuppressive drugs were tacrolimus (88.3%), mycophenolate mofetil (82.6%), and steroids (93.6%) (Table 2). The mean numbers of allograft biopsies and EU-TRAIN blood biomarkers' measurements per patient were 1.97 (±0.73) and 1.39 (± 0.74), respectively.

Detailed characteristics of participants stratified by center are provided in Table 3.

Kidney allograft phenotypes and outcome measure

A total of 812 kidney allograft biopsies were performed along with concomitant biological assessments between January 2019 and September 2021. The median time between kidney transplantation and allograft biopsy was 4.1 months (IQR, 3.0–12.1 months). The overall timeline of the biopsies is depicted in Supplementary Figure S1, with 317 (39.0%) biopsies performed at 3 months, 308 (37.9%) biopsies at 12 months, and 187 (23.0%) biopsies for clinical indication, mostly within the first 3 months after transplant (median time between kidney transplantation and clinically indicated allograft biopsies 2.04 months, IQR 0.56–6.57 months).

The cumulative incidence of rejection per patient was 8.1% (95% confidence interval [CI] = 5.7%–11%) at 1 year after transplantation. Among the 812 biopsies performed in the 412 patients included in the main analysis, rejection was diagnosed in 52, including active AMR (n = 18), chronic active AMR (n = 6), acute TCMR (n = 18), chronic active TCMR (n = 7), and mixed rejection (n = 3) (Table 2). Among these 52 rejection cases, 18 (34.6%) were subclinical (i.e., detected on protocol biopsies with a stable kidney function and no significant proteinuria). In 49 biopsies, we observed other rejection-related diagnoses, comprising borderline/suspicious for acute TCMR (n = 27), equivocal/suspicious for diagnosis of AMR (n = 9), chronic inactive AMR (n = 1), and C4d deposition without evidence of rejection (n = 12). Non–rejection-related diagnoses reflected the full spectrum of kidney transplant pathology, including minimal or no histopathologic findings (n = 513), calcineurin inhibitor toxicity (n = 29), acute tubular injury (n = 35), thrombotic microangiopathy (n = 15), BK virus nephropathy (n = 16), recurrent or *de novo* glomerulonephritis (n = 32), isolated moderate to severe interstitial fibrosis and tubular atrophy (n = 60), and other diagnoses (n = 11). Detailed characteristics of biopsies are provided in Table 2. The corresponding international Banff scores are provided in Supplementary Table S3.

Univariate association between EU-TRAIN blood biomarkers and kidney allograft rejection

Gene expression levels of *CD4* were significantly associated with allograft rejection (OR = 0.49, 95% CI = 0.32–0.73, $P < 0.001$). All other blood mRNA gene expression levels were not significantly associated with allograft rejection (Figure 3). There was a multicollinearity between blood mRNA gene expression levels (Figure 4). Their diagnostic

Table 1 | Recipient, donor, and transplant characteristics of patients included in the EU-TRAIN biomarker cohort (N = 412)

Variables	n[a]	Values
Recipient characteristics		
Age, yr, mean (SD)	412	53.4 (14.4)
Sex, n (%)	412	
Female		148 (35.9)
Male		264 (64.1)
Cause of end-stage kidney disease, n (%)	412	
ADPKD		58 (14.1)
Diabetes		41 (10.0)
Vascular		75 (18.2)
Glomerulonephritis		128 (31.1)
Tubulointerstitial		31 (7.5)
Other		41 (10.0)
Unknown		38 (9.2)
Donor characteristics		
Age, yr, mean (SD)	398	55.0 (15.1)
Blood group, n (%)	411	
A		149 (36.3)
AB		20 (4.9)
B		47 (11.4)
O		195 (47.4)
Type, n (%)	403	
Living		78 (19.4)
Deceased standard criteria donor		166 (41.2)
Deceased expanded criteria donor		159 (39.5)
Transplant baseline characteristics		
Prior kidney transplantation, n (%)	402	54 (13.4)
Cold ischemia time, h, n (%)	398	12.8 (8.2)
Delayed graft function, n (%)	412	99 (24.0)
HLA A/B/DR mismatches, median [IQR]	411	4.0 [3.0–5.0]
ABO incompatible transplantation, n (%)	412	17 (4.1)
Anti-HLA DSA at the time of transplantation, n (%)	399	82 (20.6)
Induction therapy, n (%)	412	
No induction or baliximab		172 (41.7)
Thymoglobulin		240 (58.3)

ABO, ABO blood group; ADPKD, autosomal dominant polycystic kidney disease; anti-HLA DSA, anti–human leukocyte antigen donor-specific antibody; EU-TRAIN, European TRAnsplantation and INnovation; IQR, interquartile range.
[a]Number of observations with available data regarding each variable of interest.

performances for rejection during the first year after transplantation, including discriminative ability, were low to moderate (Table 4).

The median antibody levels at the time of biopsy were 9.32 U/ml (IQR, 7.58–11.69 U/ml) for AT1R, 9.43 U/ml (IQR, 7.84–11.72 U/ml) for ETAR, 5.56 U/ml (IQR, 3.59–8.62 U/ml) for C3aR, and 11.29 U/ml (IQR, 7.05–18.20 U/ml) for C5aR. We did not find significant associations between these antibody levels and allograft rejection (Figure 5). There was a significant multicollinearity between the non-HLA antibody levels (Figure 4). Their diagnostic performances for rejection were low (AT1R antibodies: ROC-AUC = 0.55, 95% CI = 0.46–0.65, ETAR antibodies: ROC-AUC = 0.56, 95% CI = 0.47–0.66, C3aR antibodies: ROC-AUC = 0.51, 95% CI = 0.42–0.61, C5aR antibodies: ROC-AUC = 0.56, 95% CI = 0.46–0.66; see Table 5 for other metrics).

Univariate association between standard of care parameters and kidney allograft rejection

Next, we evaluated the univariate association of routine parameters (complete list available in Supplementary Table S1) with allograft rejection in the EU-TRAIN study population. The history of rejection (OR = 4.96, 95% CI = 2.37–10.39, $P < 0.001$), ABO blood group incompatibility between the recipient and donor (OR = 2.79, 95% CI = 1.11–6.97, $P = 0.03$), induction therapy with antithymocyte globulin (OR = 0.36, 95% CI = 0.20–0.65, $P = 0.001$), allograft instability at the time of biopsy (OR = 7.49, 95% CI = 4.12–13.63, $P < 0.001$), estimated glomerular filtration rate at the time of biopsy (OR = 0.96, 95% CI = 0.95–0.98, $P < 0.001$), urine protein-to-creatinine ratio at the time of biopsy (OR = 1.41, 95% CI = 1.07–1.86, $P = 0.016$), and MFI of the immunodominant anti-HLA DSA at the time of biopsy (OR = 1.56, 95% CI = 1.03–2.38, $P = 0.04$) were significantly associated with kidney allograft rejection (Figure 6). Circulating anti-HLA DSA were also strongly associated with AMR (OR = 3.20, 95% CI = 1.84–5.55, $P < 0.001$). The individual diagnostic performances of standard of care parameters are provided in Supplementary Table S4.

Additional predictive value of blood biomarkers compared with routine parameters

To assess the added value of the blood biomarkers to predict kidney allograft rejection beyond standard of care parameters, we developed and compared a model trained using solely standard of care parameters (referred to as the standard of care model), a model trained using solely blood biomarkers (referred to as the biomarker model), and a model trained using both standard of care parameters and blood biomarkers (referred to as the integrative model). Optimal combinations of predictors were selected in each model by elastic net regularized regressions. To note, 6 blood mRNA biomarkers (*AKR1C3*, *CTLA4*, *FOXP3*, *MZB1*, *POU2AF1*, and *TCL1A*) were excluded from these analyses because of a rate of missing values >30% mainly secondary to technical issues in routine monitoring.

Clinical predictors of allograft rejection in the standard of care model were allograft instability, MFI of the immunodominant anti-HLA DSA, history of rejection, induction therapy with antithymocyte globulin, delayed graft function, urine protein-to-creatinine ratio (log transformed), and estimated glomerular filtration rate (Supplementary Figure S2A). The discriminative ability of this model for allograft rejection was moderate (ROC-AUC = 0.76, 95% CI = 0.63–0.89; Figure 7).

Blood biomarkers at the time of biopsy included in the biomarker model had a low discriminative ability for allograft rejection (ROC-AUC = 0.60, 95% CI = 0.46–0.75; Figure 7).

Table 2 | Histologic findings and biological parameters at the time of kidney allograft biopsies performed during the EU-TRAIN study (N = 812)

Variables	n[a]	Values
Biopsy characteristics		
Biopsy indication, n (%)	812	
Protocol M3		317 (39.0)
Protocol M12		308 (37.9)
Clinically indicated		187 (23.0)
Main diagnosis, n (%)	812	
Active AMR		18 (2.2)
Chronic active AMR		6 (0.7)
Chronic inactive AMR		1 (0.1)
Acute TCMR		18 (2.2)
Chronic active TCMR		7 (0.9)
Mixed rejection (AMR + TCMR)		3 (0.4)
Borderline/suspicious for acute TCMR		27 (3.3)
Equivocal/suspicious for diagnosis of AMR[b]		9 (1.1)
C4d without evidence of rejection		12 (1.5)
BK virus nephropathy		16 (2.0)
Glomerulonephritis (recurrent or *de novo*)		32 (3.9)
CNI toxicity without rejection		29 (3.6)
TMA without rejection nor CNI toxicity		15 (1.8)
Acute tubular injury without rejection		35 (4.3)
Isolated IFTA ≥2		60 (7.4)
Other diagnoses		11 (1.4)
Normal or minimal changes		513 (63.2)
Biology at the time of biopsy		
eGFR (MDRD formula, ml/min per 1.73 m^2), mean (SD)	790	48.4 (20.1)
Urine protein-to-creatinine ratio, g/g, median [IQR]	662	0.2 [0.1–0.4]
Anti-HLA DSA, n (%)	772	152 (19.7)
MFI of the immunodominant anti-HLA DSA,[c] n (%)	771	
<500		620 (80.4)
500–1400		93 (12.1)
>1400		58 (7.5)
Immunosuppressive therapy at the time of biopsy		
Steroids, n (%)	787	737 (93.6)
Antimetabolite therapy, n (%)	787	
Mycophenolate mofetil		650 (82.6)
Azathioprine		48 (6.1)
No		89 (11.3)
mTOR inhibitor therapy, n (%)	784	60 (7.7)
Calcineurin inhibitor therapy, n (%)	788	
Tacrolimus		696 (88.3)
Cyclosporine		44 (5.6)
No		48 (6.1)
Belatacept, n (%)	785	47 (6.0)

AMR, antibody-mediated rejection; anti-HLA DSA, anti–human leukocyte antigen donor-specific antibody; C4d, complement component C4d; CNI, calcineurin inhibitor; DSA, donor-specific antibody; eGFR, estimated glomerular filtration rate; EU-TRAIN, European TRAnsplantation and INnovation; IFTA, interstitial fibrosis and tubular atrophy; IQR, interquartile range; MDRD, Modification of Diet in Renal Disease formula; MFI, mean fluorescence intensity; mTOR, mammalian target of rapamycin; TCMR, T cell–mediated rejection; TMA, thrombotic microangiopathy.
Clinical indications for performing a kidney allograft biopsy were deterioration of kidney function and/or detection or rise in proteinuria.
[a]Number of observations with available data regarding each variable of interest.
[b]The category "Equivocal/suspicious for diagnosis of AMR" refers to biopsies with lesions evocative of AMR but not fulfilling all the criteria for a diagnosis of AMR (moderate microvascular inflammation [g + ptc ≥ 2 in the absence of recurrent or *de novo* glomerulonephritis and at least g ≥ 1 in the presence of acute TCMR, borderline infiltrate or infection] without circulating DSA or a substitute [complement C4d staining or expression of validated molecular markers] and C4d-positive acute tubular injury without circulating DSA).
[c]Immunodominant DSA refers to the class of DSA with the highest level of fluorescence signal during measurement.

Their individual predictive importance is provided in Supplementary Figure S2B.

Finally, none of the blood biomarkers at the time of biopsy were predictors of allograft rejection in the integrative model (Supplementary Figure S2C). Moreover, the discriminative ability of this model for allograft rejection was moderate (ROC-AUC = 0.78, 95% CI = 0.67–0.88; Figure 7) and similar to the one of the standard of care model ($P = 0.45$).

Sensitivity analyses

Finally, various sensitivity analyses were performed to further confirm our findings and test the added value of blood biomarkers in different clinical scenarios and subpopulations. In all these scenarios, we confirmed the absence of incremental predictive ability of the 13 well-expressed blood mRNAs and 4 non-HLA antibodies targeting endothelial antigens to detect allograft rejection. These scenarios included (i) stable or unstable patients (i.e., with a deterioration of kidney function and/or detection or rise in proteinuria at the time of biopsy), (ii) rejection subtypes (i.e., AMR and TCMR), (iii) timing of biomarker measurement when assessed in the first 3 months after transplantation or after 3 months after transplantation, (iv) type of immunosuppressive therapy (i.e., with or without calcineurin inhibitor), (v) center effect, and (vi) kidney allograft rejection defined according to the international Banff 2022 classification[45] (Table 6).

DISCUSSION

In this large, prospective, and unselected cohort of 412 deeply phenotyped adult kidney transplant recipients with a systematic assessment of biomarkers during the first year after transplantation, the 23 EU-TRAIN blood biomarkers showed multicollinearity and did not demonstrate an additional value beyond standard of care parameters and circulating anti-HLA DSA for predicting allograft rejection.

We studied 19 mechanistically driven blood gene expression biomarkers that we and others found associated

Table 3 | Recipient, donor, and transplant characteristics of patients per center included in the EU-TRAIN cohort

Variable	N[a]	Overall (N = 412)	EU-TRAIN's transplant centers								
			Paris-Saint-Louis (n = 119)	Paris-Necker (n = 117)	Nantes (n = 43)	Barcelona-Bellvitge (n = 61)	Charité-Mitte (n = 21)	Charité-Virchow (n= 12)	Paris-Kremlin-Bicêtre (n = 20)	Barcelona-Vall d'Hebron (n = 6)	Genève (n = 13)
Recipient characteristics											
Age, yr, mean (SD)	412	53.4 (14.4)	52.3 (15.1)	52.8 (15.2)	53.4 (14.6)	55.1 (13.4)	55.0 (11.7)	49.3 (15.0)	56.1 (11.9)	62.7 (10.9)	52.0 (13.5)
Sex, n (%)	412										
Female		148 (35.9)	44 (37.0)	41 (35.0)	19 (44.2)	18 (29.5)	11 (52.4)	2 (16.7)	9 (45.0)	1 (16.7)	3 (23.1)
Male		264 (64.1)	75 (63.0)	76 (65.0)	24 (55.8)	43 (70.5)	10 (47.6)	10 (83.3)	11 (55.0)	5 (83.3)	10 (76.9)
Cause of end-stage kidney disease, n (%)	412										
ADPKD		58 (14.1)	8 (6.7)	28 (23.9)	8 (18.6)	3 (4.9)	7 (33.3)	2 (16.7)	0 (0.0)	1 (16.7)	1 (7.7)
Diabetes		41 (10.0)	18 (15.1)	6 (5.1)	1 (2.3)	10 (16.4)	0 (0.0)	1 (8.3)	3 (15.0)	2 (33.3)	0 (0.0)
Vascular		75 (18.2)	28 (23.5)	17 (14.5)	6 (14.0)	7 (11.5)	4 (19.0)	0 (0.0)	9 (45.0)	1 (16.7)	3 (23.1)
Glomerulonephritis		128 (31.1)	36 (30.3)	36 (30.8)	12 (27.9)	18 (29.5)	7 (33.3)	7 (58.3)	4 (20.0)	2 (33.3)	6 (46.2)
Tubulointerstitial		31 (7.5)	8 (6.7)	7 (6.0)	7 (16.3)	6 (9.8)	1 (4.8)	0 (0.0)	2 (10.0)	0 (0.0)	0 (0.0)
Other		41 (10.0)	6 (5.0)	15 (12.8)	7 (16.3)	8 (13.1)	0 (0.0)	1 (8.3)	1 (5.0)	0 (0.0)	3 (23.1)
Unknown		38 (9.2)	15 (12.6)	8 (6.8)	2 (4.7)	9 (14.8)	2 (9.5)	1 (8.3)	1 (5.0)	0 (0.0)	0 (0.0)
Donor characteristics											
Age, yr, mean (SD)	398	55.0 (15.1)	53.1 (16.4)	56.9 (15.0)	54.7 (14.6)	55.6 (15.2)	52.5 (14.9)	53.0 (8.2)	54.9 (11.6)	59.0 (10.8)	56.1 (12.3)
Blood group, n (%)	411										
A		149 (36.3)	40 (33.6)	36 (30.8)	19 (44.2)	29 (47.5)	2 (10.0)	6 (50.0)	9 (45.0)	3 (50.0)	5 (38.5)
AB		20 (4.9)	7 (5.9)	6 (5.1)	0 (0.0)	4 (6.6)	0 (0.0)	2 (16.7)	0 (0.0)	1 (16.7)	0 (0.0)
B		47 (11.4)	14 (11.8)	11 (9.4)	3 (7.0)	8 (13.1)	5 (25.0)	2 (16.7)	4 (20.0)	0 (0.0)	0 (0.0)
O		195 (47.4)	58 (48.7)	64 (54.7)	21 (48.8)	20 (32.8)	13 (65.0)	2 (16.7)	7 (35.0)	2 (33.3)	8 (61.5)
Type, n (%)	403										
Living		78 (19.4)	6 (5.0)	38 (32.5)	8 (18.6)	4 (6.9)	6 (30.0)	6 (85.7)	3 (15.0)	0 (0.0)	7 (53.8)
Deceased SCD		166 (41.2)	61 (51.3)	36 (30.8)	18 (41.9)	29 (50.0)	8 (40.0)	1 (14.3)	9 (45.0)	2 (33.3)	2 (15.4)
Deceased ECD		159 (39.5)	52 (43.7)	43 (36.8)	17 (39.5)	25 (43.1)	6 (30.0)	0 (0.0)	8 (40.0)	4 (66.7)	4 (30.8)
Transplant baseline characteristics											
Prior kidney transplantation, n (%)	402	54 (13.4)	22 (18.8)	10 (8.6)	4 (9.8)	10 (16.4)	0 (0.0)	2 (16.7)	2 (10.0)	2 (33.3)	2 (15.4)

(*Continued on following page*)

Table 3 | (Continued) **Recipient, donor, and transplant characteristics of patients per center included in the EU-TRAIN cohort**

			EU-TRAIN's transplant centers								
Variable	N[a]	Overall (N = 412)	Paris-Saint-Louis (n = 119)	Paris-Necker (n = 117)	Nantes (n = 43)	Barcelona-Bellvitge (n = 61)	Charité-Mitte (n = 21)	Charité-Virchow (n= 12)	Paris-Kremlin-Bicêtre (n = 20)	Barcelona-Vall d'Hebron (n = 6)	Genève (n = 13)
Cold ischemia time, h, mean (SD)	398	12.8 (8.2)	13.6 (4.7)	13.3 (10.5)	10.4 (8.3)	15.5 (8.7)	9.2 (5.8)	6.7 (6.0)	11.6 (6.5)	16.4 (6.0)	6.8 (7.5)
Delayed graft function, n (%)	412	99 (24.0)	37 (31.1)	16 (13.7)	3 (7.0)	23 (37.7)	6 (28.6)	4 (33.3)	6 (30.0)	1 (16.7)	3 (23.1)
HLA A/B/DR mismatches, median [IQR]	411	4.0 [3.0–5.0]	4.0 [3.0–5.0]	4.0 [3.0–5.0]	4.0 [3.0–5.0]	4.0 [3.0–5.0]	3.5 [2.8–5.0]	3.0 [2.8–5.0]	4.0 [3.0–5.0]	3.5 [2.3–4.0]	5.0 [3.0–5.0]
ABO incompatible transplantation, n (%)	412	17 (4.1)	1 (0.8)	5 (4.3)	1 (2.3)	1 (1.6)	0 (0.0)	4 (33.3)	2 (10.0)	1 (16.7)	2 (15.4)
Anti-HLA DSA at the time of transplantation, n (%)	399	82 (20.6)	43 (36.1)	29 (25.0)	2 (5.1)	6 (10.5)	0 (0.0)	0 (0.0)	1 (5.0)	0 (0.0)	1 (8.3)
Induction therapy, n (%)	412										
No induction or baliximab		172 (41.7)	2 (1.7)	79 (67.5)	1 (2.3)	36 (59.0)	21 (100.0)	12 (100.0)	9 (45.0)	2 (33.3)	10 (76.9)
Thymoglobulin		240 (58.3)	117 (98.3)	38 (32.5)	42 (97.7)	25 (41.0)	0 (0.0)	0 (0.0)	11 (55.0)	4 (66.7)	3 (23.1)

ABO, ABO blood group; ADPKD, autosomal dominant polycystic kidney disease; anti-HLA DSA, anti–human leukocyte antigen donor-specific antibody; ECD, expanded criteria donor; EU-TRAIN, European TRAnsplantation and INnovation; IQR, interquartile range; SCD, standard criteria donor.
[a]Number of observations with available data regarding each variable of interest.

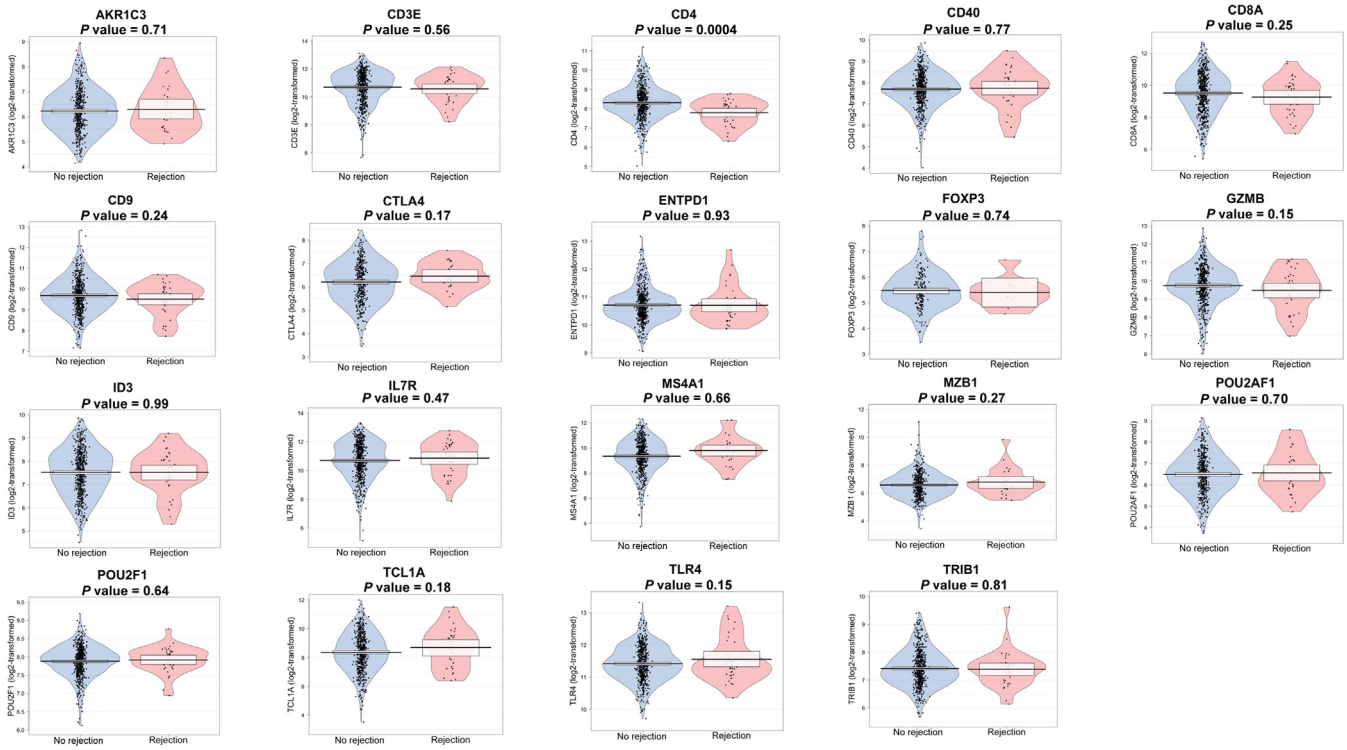

Figure 3 | Association of blood mRNAs at the time of biopsy with kidney allograft rejection. Pirate plots displaying the univariate association between blood mRNA gene expression levels at the time of biopsy and kidney allograft rejection. The horizontal bars represent medians, the bean shapes represent smooth density curves showing the full data distribution, the bands represent Bayesian highest density intervals, and the black points represent individual raw data points. Normalized counts of blood mRNAs were log2-transformed, as previously described in the literature.

with immune response, spontaneous tolerance, DSA generation, and subclinical rejection in previous studies.[6,7,15] In this unselected cohort reflecting natural disease prevalence, we demonstrate that they were not associated with allograft rejection after adjustment for standard of care parameters, including in a sensitivity analysis with only protocol biopsies (i.e., aiming to focus on subclinical rejection). Their added value combined in an integrative score with standard of care parameters remains to be further evaluated.[7]

We also assessed 4 non-HLA antibodies targeting endothelial antigens (AT1R, ETAR, C3aR receptor, and C5aR) involved in auto- and alloimmunity in multiple organ transplants.[21] Among them, AT1R and ETAR antibodies showed promising performances to discriminate kidney allograft rejection, especially AMR, and facilitated immunologic risk stratification in prior retrospective studies.[13,22] Here we show that none of them were associated with kidney allograft rejection in univariate analysis, including in a sensitivity analysis excluding TCMR and mixed rejection cases and focusing on AMR. Previous studies showed causal associations between AT1R antibody levels and rejection episodes in limited and specific scenarios.[22,23] The EU-TRAIN study assessed different contexts of use and populations, and investigated these biomarkers in a systematic prospective monitoring with a dedicated study design. The current results are consistent with a recent exploratory analysis, which indicated no association between AT1R and ETAR antibody levels and allograft rejection.[46] In addition, they align with the Sensitization in Transplantation: Assessment of Risk (STAR) working group guidelines, which do not recommend routine post-transplant assessment of non-HLA antibodies because of the insufficient evidence regarding their clinical utility.[47] Moreover, we confirmed that a proper confrontation with standard of care parameters is challenging, albeit crucial to demonstrate the clinical utility of biomarkers to inform decision-making.[25] Indeed, we demonstrated in this study that a model based solely on 7 simple clinical routine parameters (allograft instability, MFI of the immunodominant DSA, previous episode of rejection, induction therapy with antithymocyte globulin, delayed graft function, urine protein-to-creatinine ratio, and estimated glomerular filtration rate), previously established as factors associated with rejection,[48–51] resulted in a reasonable discriminative ability for allograft rejection (ROC-AUC = 0.76, 95% CI = 0.63–0.89). Among the 23 candidate EU-TRAIN blood biomarkers, none showed superior discriminative abilities, even combined in a model developed using solely biomarkers (ROC-AUC = 0.60, 95% CI = 0.46–0.75). To note, the high negative predictive values observed in this study were due to the low prevalence of rejection—which reflects our unselected design and the real-world evidence of improvement of rejection prevention with modern immunosuppressive therapies[52]—and not to good diagnostic performances (negative predictive values are

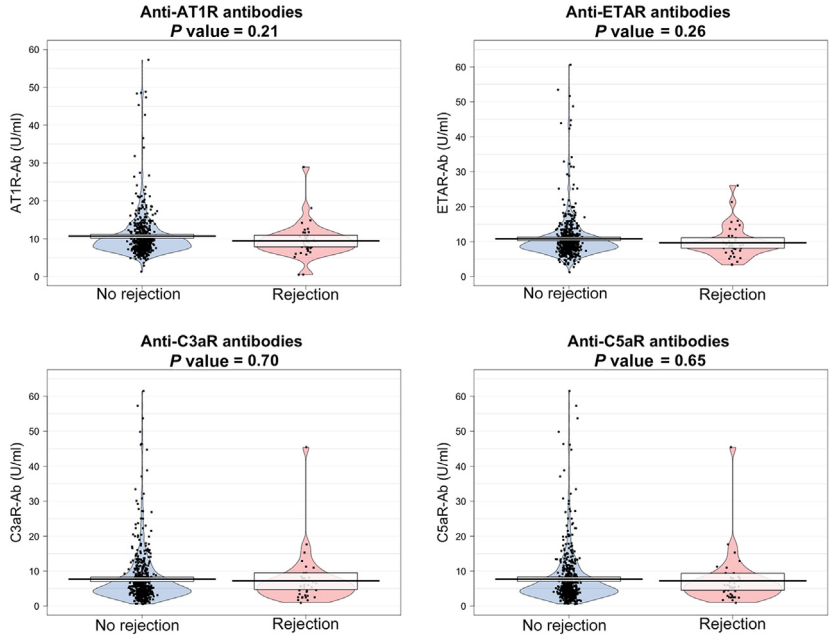

Figure 4 | Association of non–human leukocyte antigen (non-HLA) antibodies against endothelial targets at the time of biopsy with kidney allograft rejection. Pirate plots displaying the univariate association between levels of non-HLA antibodies against endothelial targets at the time of biopsy and kidney allograft rejection. The horizontal bars represent medians, the bean shapes represent smooth density curves showing the full data distribution, the bands represent Bayesian highest density intervals, and the black points represent individual raw data points. Non-HLA antibody levels were measured in U/ml. AT1R, angiotensin II receptor type 1; C3aR, complement C3a receptor; C5aR, complement C5a receptor; ETAR, endothelin 1 receptor type A.

inversely proportional to disease prevalence),[53] and thus should not be considered.

The robustness of our data results from (i) our unselected study design, which captured the real-world distribution of kidney allograft phenotypes; (ii) the confirmation of our findings in various exploratory sensitivity analyses for stable and unstable patients, subpopulations of AMR and TCMR, timing of biomarker measurement after transplantation, type of immunosuppressive therapy, across centers, and definition of kidney allograft rejection according to the international

Table 4 | Diagnostic performances of blood mRNA gene expression levels at the time of biopsy to detect kidney allograft rejection

Blood mRNA biomarkers	Discriminative ability, ROC-AUC (95% CI)	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)
AKR1C3	0.51 (0.39–0.63)	92.31	15.58	5.31	97.53
CD3E	0.55 (0.46–0.64)	80.00	37.27	7.00	96.93
CD4	0.68 (0.60–0.76)	77.14	52.95	8.82	97.52
CD40	0.52 (0.42–0.63)	54.55	57.78	6.79	95.75
CD8A	0.56 (0.47–0.66)	82.86	34.18	6.94	97.12
CD9	0.54 (0.44–0.63)	91.43	22.02	6.45	97.76
CTLA4	0.59 (0.49–0.69)	83.33	40.26	7.91	97.52
ENTPD1	0.54 (0.43–0.64)	62.86	48.66	6.71	95.71
FOXP3	0.53 (0.36–0.73)	88.89	35.16	6.35	98.46
GZMB	0.57 (0.46–0.67)	22.86	91.84	14.29	95.24
ID3	0.50 (0.40–0.59)	80.00	27.35	6.29	95.73
IL7R	0.53 (0.44–0.63)	68.57	43.34	6.67	95.90
MS4A1	0.58 (0.48–0.67)	81.25	37.52	6.77	97.29
MZB1	0.54 (0.43–0.66)	75.00	38.35	5.68	96.88
POU2AF1	0.50 (0.39–0.62)	19.35	90.91	12.00	94.62
POU2F1	0.52 (0.43–0.61)	85.71	27.73	6.52	97.06
TCL1A	0.56 (0.48–0.68)	43.33	75.09	8.61	96.07
TLR4	0.53 (0.42–0.63)	25.71	89.95	13.04	95.38
TRIB1	0.51 (0.42–0.60)	78.79	34.86	6.36	96.70

CI, confidence interval; ROC-AUC, area under the receiver operating characteristic curve.

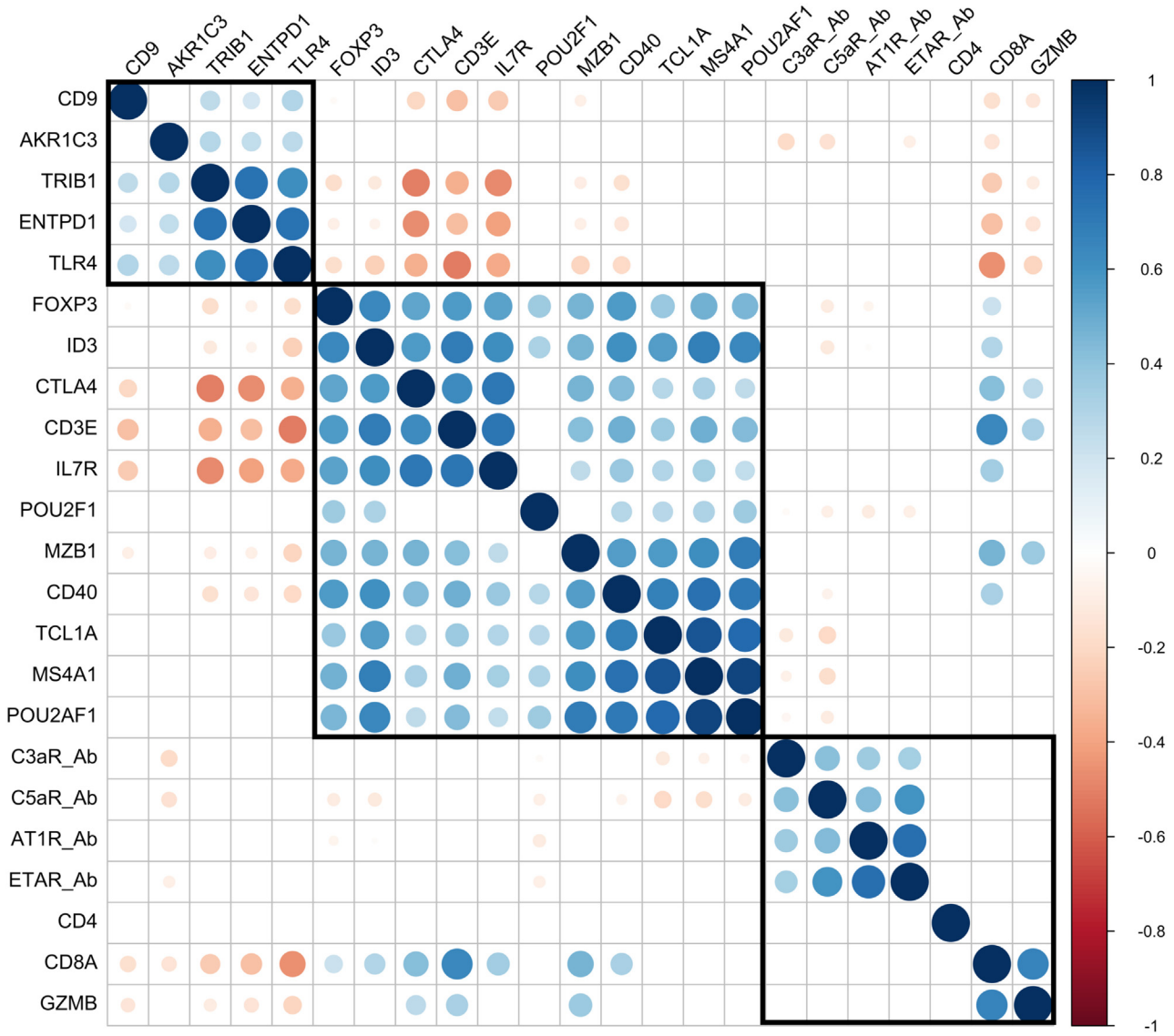

Figure 5| Multicollinearity between EU-TRAIN, European TRAnsplantation and INnovation blood biomarkers. This correlation matrix shows the relationship between the values of blood biomarkers. Only statistically significant correlations are represented in each box. Positive correlations are in blue and negative correlations are in red. The diameter of each correlation circle is proportional to the strength of the correlation. The clusters of correlations are framed in black. AT1R_Ab, antibodies against angiotensin II receptor type 1; C3aR_Ab, antibodies against complement C3a receptor; C5aR_Ab, antibodies against complement C5a receptor; ETAR_Ab, antibodies against endothelin 1 receptor type 1.

Table 5| Diagnostic performances of non-HLA antibodies against endothelial targets at biopsy to detect kidney allograft rejection

Non-HLA antibodies against	Discriminative ability, ROC-AUC (95% CI)	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)
AT1R	0.55 (0.46–0.65)				
Low threshold (10 U/ml)	–	38.46	58.38	6.22	92.96
High threshold (17 U/ml)	–	5.13	92.08	4.44	93.11
ETAR (threshold 10 U/ml)	0.56 (0.47–0.66)	30.77	57.46	4.94	92.04
C3aR	0.51 (0.42–0.61)	25.64	83.06	9.80	93.96
C35aR	0.56 (0.46–0.66)	53.85	59.67	8.75	94.74

AT1R, angiotensin II receptor type I; C3aR, complement C34a receptor; C5aR, complement C5a receptor; CI, confidence interval; ETAR, endothelin receptor 1 receptor type A; HLA, human leukocyte antigen; ROC-AUC, area under the receiver operating characteristic curve.
Positive thresholds are based on the literature for AT1R and ETAR antibodies and calculated with the Youden index for C3aR and C5aR antibodies (as there is no threshold published in previous studies for these last 2 biomarkers).

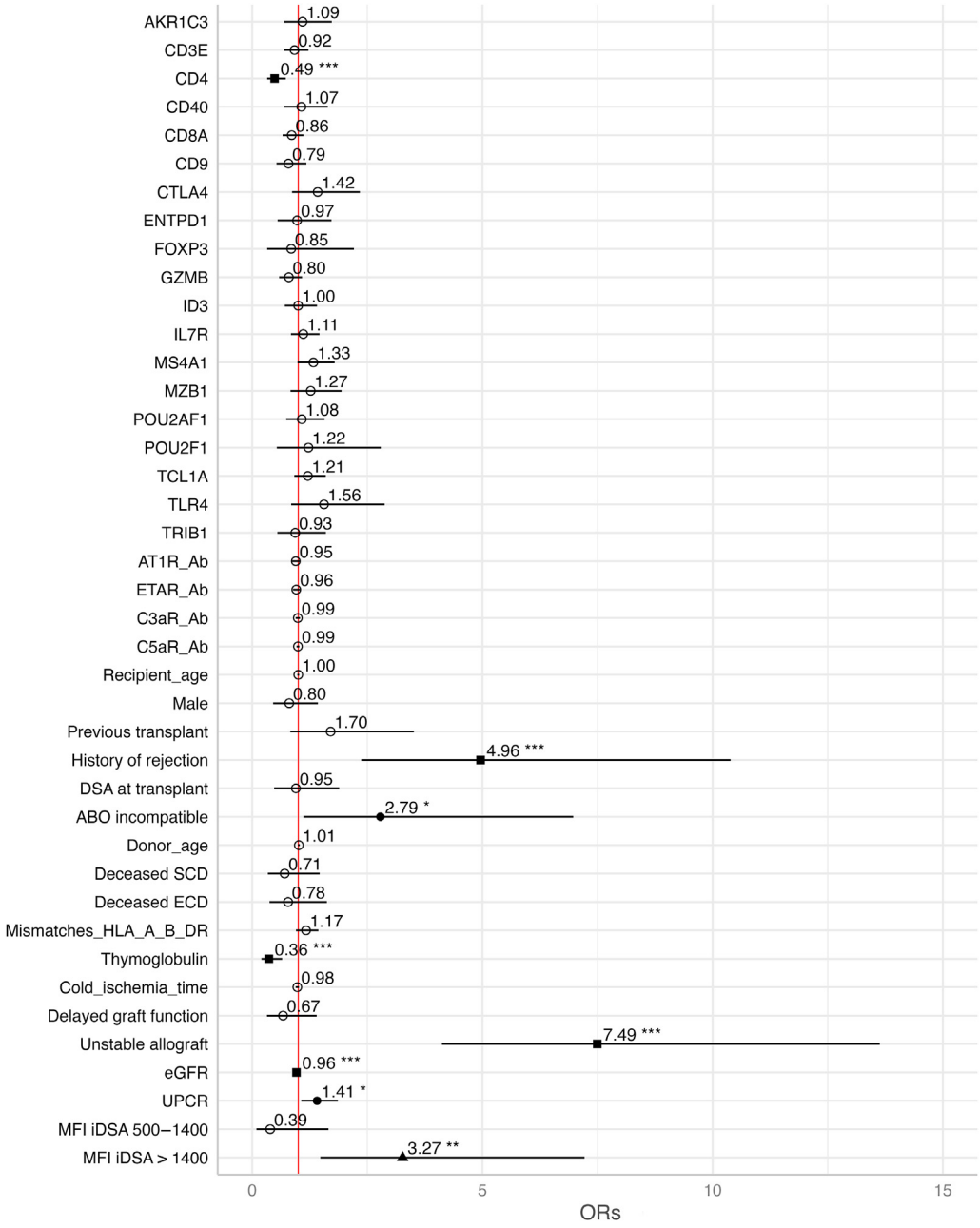

Figure 6 | Association of emerging blood biomarkers and standard of care parameters at the time of biopsy with kidney allograft rejection. This forest plot shows the univariate association of blood mRNA gene expression, non-HLA antibodies, and routinely collected parameters with kidney allograft rejection. The numbers correspond to odds ratios (ORs) and the horizontal lines correspond to 95% confidence intervals of the respective odds ratios. Statistically significant associations are represented by black squares (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$), and nonsignificant associations are represented by transparent circles. AT1R_Ab, antibodies against angiotensin II receptor type 1; C3aR_Ab, antibodies against complement C3a receptor; C5aR_Ab, antibodies against complement C5a receptor; DSA, donor-specific antibody; ECD, expanded criteria donor; eGFR, estimated glomerular filtration rate; ETAR_Ab, antibodies against endothelin 1 receptor type 1; iDSA, immunodominant DSA; MFI, mean fluorescence intensity; non-HLA, non–human leukocyte antigen; SCD, standard criteria donor; UPCR, urine protein-to-creatinine ratio.

Banff 2022 classification;[45] and (iii) the use of elastic net regularized regressions, which allow us to generate the optimal combinations of blood biomarkers to increase predictive performances of the diagnostic models, showing that even combined optimally, they did not have an incremental value beyond standard of care parameters.

Finally, our data show that unselected prospective cohort studies are a critical step to validate the clinical utility of monitoring biomarkers, before moving toward implementation in clinical care.[25] Indeed, unselected studies allow to capture natural disease prevalence and correctly assess biomarkers in settings that mimic real life and capture the full

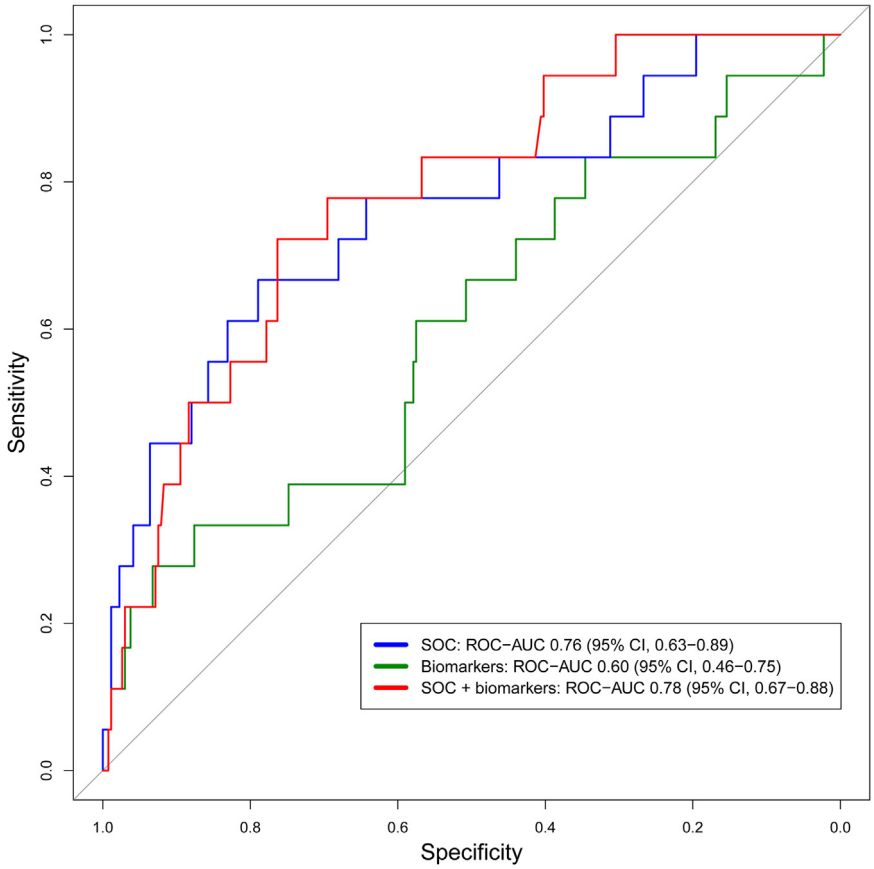

Figure 7 | Comparison of the performances of the predictive models of kidney allograft rejection developed using blood biomarkers, routine parameters, or all parameters. Discriminative abilities of the standard of care (SOC) model, biomarker model, and integrative model (SOC + biomarkers) to detect kidney allograft rejection, assessed through the calculation of the area under the receiver operating characteristic curve (ROC-AUC). To note, 6 blood mRNA biomarkers (*AKR1C3*, *CTLA4*, *FOXP3*, *MZB1*, *POU2AF1*, and *TCL1A*) were excluded from these analyses because of a rate of missing values >30% mainly secondary to technical issues in routine monitoring. CI, confidence interval.

spectrum of kidney allograft phenotypes.[25] For instance, the blood gene expression assay, named kidney Solid Organ Response Test (kSORT), showed promising diagnostic performances in case-control proof of concept studies, but then failed to be clinically validated in an unselected cohort.[54,55] Still, these results could be used for health technology assessment and integrated into *de novo* decision models in a broad spectrum of clinical settings and national contexts.[56,57] However, conducting such studies is challenging[58] and requires, for instance, for this work joint efforts of a multisite kidney transplant network including a range of researchers, immunologists, nephrologists, pathologists, pharmacologists, statisticians, public health authorities, and industrial partners. Hence, because of time and financial constraints, biomarkers are often initially assessed in retrospective selected case-control studies, which do not reflect natural disease prevalence and are thus at high risk of bias.[26,27] Here, our robust design allowed us to demonstrate that the panel of blood mRNAs and non-HLA antibodies assessed in this study were not significantly associated with allograft rejection in the first year after transplantation, and displayed a multicollinearity reflecting that they share a significant biological redundancy.[25,59] Indeed, most of these biomarkers have been described in relation to immune-related mechanisms. B cell–related genes were associated with allograft tolerance and others,[57,58] such as *FOXP3* and *CTLA4*, are well recognized in immune tolerance.[6] This shows that these genes are modulated in specific immune-related situations and may imply specific contexts of use, such as *AKR1C3* and *TCL1A*, in operational tolerance and subclinical AMR.[7,27] This suggests that these results may not be generalized for all events and at any time after transplantation.

Our study has limitations. First, we followed patients only during the first year after transplantation; hence, our results are valid in this context of use but are not generalizable beyond this period. In contrast, in this study, most biopsies were analyzed early after transplantation (median time of 4.1 months after transplantation). Because mRNA parameters measured in total blood are sensitive to cell composition, their levels may be altered by perioperative treatments and induction therapies. Secondly, although our cohort was multicentric, all the patients were European, and we did not have access to patient ethnicity data. Thirdly, the cumulative incidence of the primary outcome (allograft rejection) was

Table 6 | Sensitivity analyses in different clinical scenarios and subpopulations

Clinical scenarios and subpopulations	Biopsies, n	Events, n	ROC-AUC (95% CI)
In stable allografts	625	18	
Biomarkers alone			0.58 (0.47–0.69)
SOC alone			0.70 (0.56–0.83)
SOC + biomarkers (integrative model)			0.72 (0.59–0.84)
In unstable allografts	187	34	
Biomarkers alone			0.67 (0.56–0.78)
SOC alone			0.77 (0.69–0.85)
SOC + biomarkers (integrative model)			0.69 (0.60–0.78)
In biopsies with AMR	784	24	
Biomarkers alone			0.66 (0.54–0.77)
SOC alone			0.78 (0.67–0.89)
SOC + biomarkers (integrative model)			0.77 (0.68–0.87)
In biopsies with TCMR	785	25	
Biomarkers alone			0.72 (0.62–0.82)
SOC alone			0.85 (0.77–0.93)
SOC + biomarkers (integrative model)			0.85 (0.77–0.93)
In biopsies <3 months after transplantation	200	23	
Biomarkers alone			0.71 (0.59–0.83)
SOC alone			0.80 (0.69–0.90)
SOC + biomarkers (integrative model)			0.77 (0.67–0.86)
In biopsies ≥3 months after transplantation	612	29	
Biomarkers alone			0.62 (0.51–0.72)
SOC alone			0.81 (0.71–0.90)
SOC + biomarkers (integrative model)			0.81 (0.72–0.90)
CNI therapy at the time of biopsy	740	45	
Biomarkers alone			0.69 (0.62–0.77)
SOC alone			0.83 (0.76–0.90)
SOC + biomarkers (integrative model)			0.83 (0.77–0.89)
No CNI therapy at the time of biopsy	44	4	
Biomarkers alone			0.61 (0.30–0.93)
SOC alone			0.68 (0.39–0.97)
SOC + biomarkers (integrative model)			0.68 (0.36–0.99)
In French centers[a]	629	30	
Biomarkers alone			0.70 (0.59–0.80)
SOC alone			0.82 (0.73–0.91)
SOC + biomarkers (integrative model)			0.82 (0.73–0.90)
In non-French centers[b]	161	22	
Biomarkers alone			0.62 (0.49–0.75)
SOC alone			0.77 (0.66–0.88)
SOC + biomarkers (integrative model)			0.75 (0.64–0.86)
Allograft rejection defined according to the international Banff 2022 classification[45]	812	61	
Biomarkers alone			0.55 (0.46–0.66)
SOC alone			0.80 (0.68–0.92)
SOC + biomarkers (integrative model)			0.80 (0.68–0.91)

AMR, antibody-mediated rejection; CI, confidence interval; CNI, calcineurin inhibitor; EU-TRAIN, European TRAnsplantation and INnovation; ROC-AUC, area under the receiver operating characteristic curve; SOC, standard of care; TCMR, T cell–mediated rejection.

[a]Paris-Saint-Louis, Paris-Necker, Nantes, and Paris-Kremlin-Bicêtre.

[b]Barcelona-Bellvitge, Barcelona-Vall d'Hebron, Berlin-Charité Mitte, Berlin-Charité Virchow, and Geneva.

We applied the locked parameters of the EU-TRAIN biomarker model (developed using only blood biomarkers), SOC model (developed using only routine parameters), and integrative model (developed using blood biomarkers and routine parameters) in different scenarios and subpopulations to assess their discriminative abilities to detect kidney allograft rejection in these settings. Allograft instability was defined as a 20% increase in serum creatinine and/or a worsening or appearance of a urine protein-to-creatinine ratio ≥0.5 g/g at the time of biopsy.

low (8.1% at 1 year after transplantation). However, it was in line with epidemiologic data gathered in the modern era of immunosuppression,[52] and we included the target number patients, calculated *a priori*, to have a sufficient statistical power. Fourthly, although there was not any statistical tendency for an additional value of the EU-TRAIN blood biomarkers in the various exploratory sensitivity analyses, this study was not specifically designed to address these outcomes. Finally, our analysis was restricted to 23 candidate blood biomarkers, but we constituted a large biobank of blood samples, which will be stored for 30 years for potential future biomarker validation studies. Furthermore, we experienced a high number of missing values for some genes (*AKR1C3*, *CTLA4*, *FOXP3*, *MZB1*, *POU2AF1*, and *TCL1A*), which may logically be related to the low level of expressing cells in total blood, such as regulatory T cells for *FOXP3*. For these lowly expressed genes, the use of conventional quantitative polymerase chain reaction with enzymatic amplification may have reduced the number of missing values and increased measure precision for these genes close to the limit of detection with the multiplex gene expression method used. None of these 6 genes showed statistical associations with allograft rejection in univariate analysis.

In conclusion, we demonstrate that the 23 EU-TRAIN blood biomarkers of gene expression profiling and non-HLA antibodies targeting endothelial antigens assessed in this large prospective multicenter study were not significantly associated with kidney allograft rejection. This questions their clinical utility for monitoring rejection in adult kidney transplant recipients during the first year after transplantation and justifies their evaluation in specific contexts of use. The EU-TRAIN unselected and multimodal design paves the way

for future studies assessing the relevance of biomarkers for clinical decision-making in kidney transplantation.

DISCLOSURE

RD and SB have ownership interests in the BioMAdvanced Diagnostics company, which is not involved in the present research. HH is chief executive officer of CellTrend. AL holds shares in Predict4Health, a software company that is not involved in the present research. All the other authors declared no competing interests.

DATA STATEMENT

Data are available from the corresponding author on reasonable request, including standards for General Data Protection Regulation and approval by the Ethics Committee of the EU-TRAIN's consortium.

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Additional information

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AUTHOR CONTRIBUTIONS

AL, CLef, OB, DD, and KB designed the EU-TRAIN study. VG, RD, RAC, MRac, AP, ME, MRay, OA, DB, FG, EV, SY, JD, HH, JLT, CRL, MZ, EP, HLM, TVHN, FM, TB, JV, CLef, DD, VP, LP, MG, PAG, SB, EC, FH, KB, OB, AL and CLef participated in the building of the EU-TRAIN cohort and contributed to data acquisition. VG, MRac, MRay, OA, AL, and CLef performed data analysis. VG, RD, RAC, MRac, MRay, OA, PAG, SB, KB, OB, AL, and CLef performed data interpretation. VG, MRac, AL, and CLef wrote the first draft of the manuscript. All authors revised and critically reviewed the manuscript.

Supplementary material is available online at www.kidney-international.org.

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