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BRIEF COMMUNICATION

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# Adequate tacrolimus exposure modulates the impact of HLA class II molecular mismatch: a validation study in an American cohort

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Clinicians have few tools to predict the risk of alloimmune injury that would guide immunosuppression management in renal transplant patients. We evaluated human leukocyte antigen (HLA)-DR/DQ molecular mismatch to predict de novo donor-specific antibodies (DSAs) during the first year of transplant and explored how differences in tacrolimus exposure may modulate this risk. HLA-DR and -DQ eplet mismatches were determined between 444 donor-recipient pairs in Denver, Colorado between 2007 and 2013. Previously defined mismatch thresholds stratified recipients into low- (N = 119), intermediate- (N = 153), and high- (N = 172) risk categories. The area under the curve for DSA at 1 year was 0.84 and 0.82 for HLA-DR and HLA-DQ eplet mismatches, respectively. Compared to low-risk patients, there was a graded increase in risk of DR/DQ DSA in intermediate (HR 15.39, 95% CI 2.01-118.09, $p$ = .009) and high-risk (HR 23.81, 95% CI 3.17-178.66, $p$ = 0.002) categories. Intermediate- and high-risk patients with a mean tacrolimus <6 ng/ml versus >8 ng/ml had increased risk of DR/DQ DSA at 1 year (HR 2.34, 95% CI 1.05-5.22, $p$ = .04). HLA molecular mismatch represents a reproducible, objective, and clinically relevant tool to stratify patients by alloimmune risk and may help guide personalized immunosuppression management.

**KEYWORDS**
alloantibody, autoimmunity, clinical research / practice, histocompatibility, immunosuppressant - calcineurin inhibitor: tacrolimus, immunosuppression / immune modulation, kidney transplantation / nephrology, monitoring: immune, rejection

## 1 | INTRODUCTION

Patients undergoing kidney transplantation are subject to an array of side effects and complications associated with the immunosuppressive therapies required for successful engraftment of the organ. The amount of immunosuppression needed is largely dependent upon the underlying immunologic risk of the patient. Currently, the assessment of this risk relies on a collection of demographic or clinical variables that often lack precision, including age, ethnicity, sensitization, type of donor kidney, and whole antigen mismatch.[1] Consequently, most decisions about induction therapy and maintenance immunosuppression are dictated by center protocol rather than the immunologic risk

profile of a given patient, with subsequent changes to the maintenance regimen carried out only after complications have occurred (eg, infection, neurotoxicity, diarrhea, etc.).[2,3] In an effort to reduce drug toxicities, some centers have advocated for minimizing exposure to tacrolimus, the most important therapy for maintenance immunosuppression in clinical practice. However, there is now a growing body of evidence suggesting this strategy may be associated with de novo donor-specific antibody (DSA) development.[4–6] The appearance of DSA, particularly those specific for human leukocyte antigen (HLA)-DR and -DQ loci, frequently evolves into chronic antibody-mediated rejection, a leading cause of renal allograft loss.[7]

Tissue matching is central to understanding a given patient's immunologic risk, as evidenced by the low immunosuppressive requirement and excellent prognosis of a kidney from a sibling that is a two haplotype HLA match. However, traditional whole antigen matching at HLA-A, -B, and -DR is only a modest predictor of adverse clinical outcomes, as a binary "match" or "mismatch" at any given HLA loci can drastically overlook similarities or differences between recipient and donor tissue at the molecular level of the HLA molecule. Small clusters of amino acids on the surface of HLA molecules referred to as "eplets" can now be compared between donor and recipient tissues in order to quantify differences in a more precise way.[8] Preliminary evidence in a single cohort of Canadian patients demonstrated that HLA-DR/DQ molecular mismatch thresholds could be used to risk stratify patients into groups that are at low, intermediate, and high risk for the development of HLA Class II antibodies.[9]

The purpose of this study is to validate the utility of molecular mismatching to predict the risk of early anti-HLA-DR and -DQ DSA development and whether it might offer a more personalized approach to immunosuppression management. Specifically, we evaluate a cohort of patients maintained on a tacrolimus minimization protocol during the critically important time early in the posttransplant course, exploring the accuracy and reproducibility of previously defined molecular mismatch thresholds to traditional measures of immunologic risk and how differences in tacrolimus exposure may impact these alloimmune risk categories on the development of DSAs.

## 2 | METHODS

### 2.1 | Study population

We included all adult patients (≥18 years old) receiving a kidney or kidney/pancreas transplant at the University of Colorado Hospital between September 2007 and December 2013 who were initiated on tacrolimus as part of triple maintenance immunosuppression therapy at the time of kidney transplantation. Patients were excluded if they had simultaneous liver and kidney transplant, previous islet cell transplant, had pretransplant DSAs, failed to undergo DSA screening, or did not have enough donor sample to perform HLA typing. This study was conducted in accordance with the Declaration of Helsinki and was approved by the ethics committee at the University of Colorado (19-0367).

### 2.2 | Immunosuppression

Induction therapy with rabbit anti-thymocyte globulin was utilized for living nonrelated kidney and kidney/pancreas transplant recipients and patients with pretransplant calculated panel reactive antibodies >20%, repeat transplant, African American race, or cold ischemia time >24 h. Patients were placed on tacrolimus, mycophenolate, and steroids with a minority receiving mammalian target of rapamycin inhibitor in place of mycophenolate. Target tacrolimus trough concentrations per center protocol were 6 – 9 ng/ml for months 0 – 3 and 5 – 8 ng/ml for months 4 – 12.

### 2.3 | HLA typing and pre- and posttransplant antibody screening

Recipient and donor HLA-DQ and -DR loci were typed by sequence-specific oligonucleotide (LABType SSO DRB1 [XR], DRB345, and DQA1/B1, One Lambda, West Hills, CA) and supplemental sequence specific primer technology as necessary (additional detail in Supplemental Methods). The single molecule eplet mismatch scores were calculated for each HLA-DR$\beta_{1/3/4/5}$ and HLA-DQ$_{\alpha/\beta}$ donor molecule using HLAMatchmaker DRDQDP (version 2). To move the individual HLA-DR and HLA-DQ molecular mismatch scores into a prognostic biomarker, a *combined (or composite)* HLA-DR/DQ molecular mismatch score was developed based on the greatest risk associated with the patient's individual HLA-DR and HLA-DQ mismatch scores. Derivation of molecular mismatch risk stratification using HLA-DR$\beta_{1/3/4/5}$ and HLA-DQ$_{\alpha/\beta}$ single molecule specific thresholds is summarized in Table 1 and described in more detail in the supplemental methods.[8] All HLA testing and antibody analyses were reviewed by ABHI board-certified HLA specialists in an ASHI- and CAP-accredited laboratory (ClinImmune Labs, Aurora, CO). Kidney and kidney/pancreas transplant recipients on the waiting list were screened for pretransplant HLA antibodies monthly. At the time of transplant, flow cytometry crossmatching was performed on all available sera up to 6 months prior to the transplant date. Following transplant, DSA screening was performed at 1, 6, 12 months, and when clinically indicated by graft dysfunction. Patients were screened for both class I and class II antibodies using

**TABLE 1** Risk of Primary Alloimmunity by Single Molecule Eplet Mismatch Thresholds

| Risk for primary alloimmunity | Single molecule eplet mismatch | | |
|---|---|---|---|
| | HLA-DR$\beta_{1/3/4/5}$ | | HLA-DQ$_{\alpha/\beta}$ |
| Low | 0-6 | and | 0-8 |
| Intermediate | 0-21 | and | 9-14 |
| High | 0-21 | and | $\geq$ 15 |

LABScreen Mixed beads (One Lambda). Positive tests were quantified using single antigen beads (One Lambda) per the manufacturer's specifications. Serum samples were treated with dithiothreitol to remove inhibition. DSAs were defined as an absolute mean florescence intensity (MFI) of >500 and at least 2 standard deviations (SDs) above the mean of the negative control serum. All MFI were normalized against negative control beads per manufacturer's instructions.

### 2.4 | Statistical methods

Variables were summarized using mean and SD or count and proportion. Comparisons of baseline characteristics by immunologic risk category were performed using one-way ANOVA, Chi-square, or Fisher's exact tests as appropriate. Receiver operating characteristic (ROC) analysis was used to validate HLA-DR$\beta_{1/3/4/5}$ and HLA-DQ$_{\alpha/\beta}$ molecule single molecule specific thresholds ≥7 of HLA-DR and ≥9 for HLA-DQ in this cohort (Figure 1). Time to DR/DQ was defined as the time from transplant to DR/DQ DSAs, if antibodies occurred within 1 year. Patients who did not develop DR/DQ DSAs within 1 year were censored at the last follow-up or 13 months posttransplant, whichever was earliest. Death prior to antibody development was treated as a competing risk. Tacrolimus trough levels were summarized as the average level between transplant and DR/DQ DSAs or censoring time, whichever came first. Average tacrolimus trough was categorized as (1) <6.0 ng/ml, (2) 6.0-7.9 ng/ml, and (3) ≥8.0 ng/ml. Cumulative incidence curves were fit for the marginal event of time-to-DR/DQ DSAs, stratified by immunologic risk category. Tests across strata were performed using Gray's test. Proportional subdistribution hazards models were fit to time-to-DR/DQ DSAs with death as a competing risk.[10] Univariate models were fit with each of the following covariates: immunologic risk category, age at transplant, sex, ethnic group, prior transplant, donor type, thymoglobulin use, mycophenolate dose reduction within 1 year, steroid type, delayed graft function, and average tacrolimus level. Any variables that were loosely associated with time-to-DSAs in univariate modeling ($p < 0.20$) were considered in the multivariable model. The final model was chosen based on the lowest Akaike Information Criterion. R version 3.6.0 was used for all analysis, and the significance level was set at 0.05.

## 3 | RESULTS

### 3.1 | Demographics and baseline characteristics

There were 444 kidney and kidney/pancreas recipients who met criteria for the analysis after exclusion for simultaneous liver and kidney transplant (N = 19), previous islet cell transplant (N = 5),

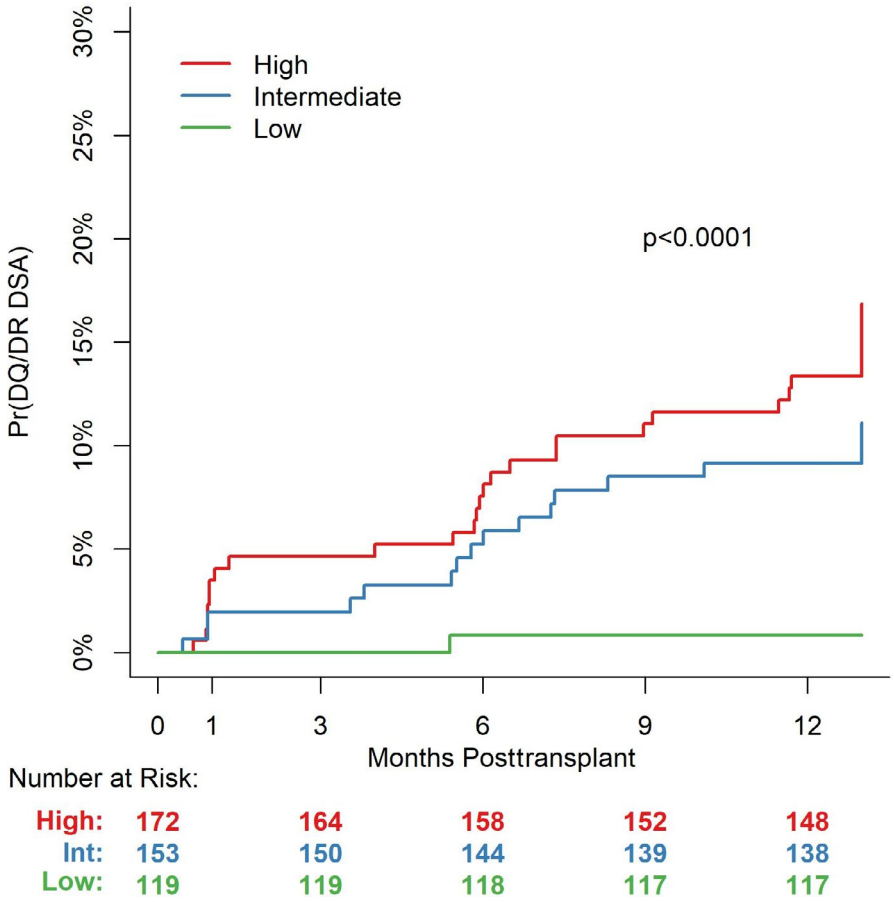


**FIGURE 1** The distribution of time-to-DR/DQ de novo donor-specific antibodies differed significantly by immunologic risk category [Color figure can be viewed at wileyonlinelibrary.com]

**TABLE 2** Baseline Characteristics of Study Population According to Molecular Mismatch Categorization

| Characteristic | Molecular mismatch risk category | | | *p* value |
|---|---|---|---|---|
| | Low (n = 119) | Intermediate (n = 153) | High (n = 172) | |
| Age, years | 48.66 ± 14.12 | 49.95 ± 13.37 | 49.09 ± 13.05 | .7158 |
| Male sex, no. (%) | 72 (61) | 91 (59) | 113 (66) | .4672 |
| Ethnicity, no. (%) | | | | .015 |
| Caucasian | 98 (82) | 96 (63) | 122 (71) | |
| Hispanic | 12 (10) | 36 (24) | 25 (15) | |
| African American | 7 (6) | 17 (11) | 16 (9) | |
| Other | 2 (2) | 4 (3) | 9 (5) | |
| Etiology of native chronic kidney disease, no. (%) | | | | .2381 |
| Diabetes | 28 (24) | 47 (31) | 65 (38) | |
| Glomerulonephritis | 46 (39) | 48 (31) | 47 (27) | |
| Hypertension | 15 (13) | 18 (12) | 17 (10) | |
| Other | 30 (25) | 40 (26) | 43 (25) | |
| Repeat transplant, no. (%) | 17 (14) | 10 (7) | 9 (5) | .0142 |
| Whole antigen mismatch, no. (%) | | | | <.0001 |
| 0 DR +DQ mismatches | 50 (42) | 0 (0) | 0 (0) | |
| 1 – 2 DR +DQ mismatches | 55 (46) | 68 (44) | 58 (34) | |
| 3 – 4 DR +DQ mismatches | 14 (12) | 85 (56) | 114 (6) | |
| Deceased donor, no. (%) | 64 (54) | 84 (55) | 88 (51) | .7865 |
| Female donor, no. (%) | 56 (50) | 66 (45) | 77 (46) | .7766 |
| Donor age, years | 38.89 ± 12.41 | 40.49 ± 12.55 | 38.07 ± 13.99 | .2622 |
| Cold ischemic time, hours | 7.99 ± 10.32 | 7.87 ± 7.78 | 8.9 ± 9.72 | .5775 |
| Delayed graft function, no. (%) | 9 (8) | 11 (7) | 12 (7) | .982 |
| Induction therapy | | | | .1216 |
| Thymoglobulin, no. (%) | 50 (42) | 75 (49) | 97 (56) | |
| Steroid only, no. (%) | 65 (55) | 70 (46) | 67 (39) | |
| Interleukin−2 inhibitor or other, no. (%) | 4 (3) | 8 (5) | 8 (5) | |
| Antimetabolite therapy | | | | .3226 |
| Mycophenolate, no. (%) | 110 (92) | 143 (93) | 153 (89) | |
| Mammalian target of rapamycin inhibitor, no. (%) | 7 (6) | 8 (5) | 18 (10) | |
| History of mycophenolate reduction, no. (%) | 26 (22) | 32 (21) | 40 (23) | .8769 |
| Mean tacrolimus by 12 months (ng/ml) | 7.22 ± 1.21 | 7.25 ± 1.42 | 7.29 ± 1.3 | .8948 |
| Mean tacrolimus by 12 months, no. (%) | | | | .6854 |
| <6.0 ng/ml | 16 (13) | 25 (16) | 25 (15) | |
| 6.0-7.9 ng/ml | 76 (64) | 90 (59) | 97 (56) | |
| ≥8.0 ng/ml | 27 (23) | 38 (25) | 50 (2) | |
| Acute cellular rejection prior to DSA, no. (%) | 5 (4) | 7 (5) | 13 (8) | .3714 |

pretransplant DSAs (N = 272), inadequate DSA screening, and inadequate donor sample to perform HLA typing (N = 212). Baseline characteristics for all patients are shown in Table 2. The cohort had a similar percentage of Caucasian recipients compared to the original Canadian cohort (Canadian cohort 65%, Denver cohort 71%). However, the Canadian cohort had far more recipients of Asian (13%) and Indigenous (20%) decent, whereas the Denver cohort had more recipients of Hispanic decent (16%) and African American (9%) decent. Fifty percent of patients received thymoglobulin induction, 45.5% had steroid-only induction, and 4.5% received other induction agents that consisted almost entirely of IL-2 receptor inhibitors. Most patients received tacrolimus, mycophenolate, and prednisone with a minority of patients receiving tacrolimus, a mammalian target of rapamycin inhibitor, and prednisone (7.4%).

**TABLE 3** Multivariable Analysis for Risk of DR/DQ de novo Donor-Specific Antibodies by 12 Months Posttransplant (N = 444)

| Predictor | Hazard ratio (95% CI) | *p* value |
|---|---|---|
| Molecular mismatch risk category (reference=low risk) | | |
| Intermediate risk | 15.39 (2.01-118.09) | .009 |
| High risk | 23.81 (3.17-178.66) | .002 |
| Average tacrolimus, months 0-12 (reference ≥8.0 ng/ml) | | |
| <6.0 ng/ml | 2.34 (1.05-5.22) | .04 |
| 6.0-7.9 ng/ml | 1.09 (0.54-2.18) | .81 |
| Age at transplant, years | 0.96 (0.94-0.98) | .0001 |
| Deceased donor | 2.74 (1.47-5.1) | .002 |

Abbreviation: CI, confidence interval.

In the entire cohort, 82 (18.5%) patients developed DSA by 12 months. Twenty-six (5.9%) patients developed either HLA-A or HLA-B de novo DSA and 9 patients (2%) developed HLA-C or HLA-DP de novo DSA by 12 months. Forty-seven patients (10.6%) developed HLA-DR or HLA-DQ DSA by 12 months with 11 at 1 month, 20 at 6 months, and 16 at 12 months (median 6.0, IQR 3.7-10.8 months). Of all DR/DQ de novo DSA, 20 (42.6%) were DQ alone, 7 (14.9%) DR alone, and 20 (42.6%) mixed DR/DQ antibodies. Among patients with DR/DQ de novo DSA, 7 (14.8%) patients had an MFI 500-999, 20 (42.6%) patients had an MFI 1000 – 2999, 14 (29.8%) patients with an MFI >3000 – 9999, and 6 (12.8%) patients with an MFI ≥10 000.

## 3.2 | Molecular mismatch and risk of donor-specific antibodies

Using previously defined HLA-DR/DQ molecular mismatch thresholds (Table 1), 444 patients were categorized as low molecular mismatch risk (n = 119, 26.8%), intermediate molecular mismatch risk (n = 153, 34.5%), and high molecular mismatch risk (n = 172, 38.7%) for DR/DQ DSA development. The HLA-DR$\beta_{1/3/4/5}$ single molecule eplet mismatch threshold of ≥7 associated with an area under the curve (AUC) of 0.84 with a sensitivity of 100% and specificity of 69% for development of DR DSA. HLA-DQ$\alpha_1\beta_1$ eplet mismatch threshold of ≥9 associated with an AUC of 0.82 with a sensitivity of 96% and specificity of 58% for development of DQ DSA (Figure S1, Tables S1 and S2).

Of the 47 patients who developed DR/DQ DSAs, there was 1 (2%) low molecular mismatch risk patient, 17 (36%) intermediate molecular mismatch risk patients, and 29 (62%) high molecular mismatch risk patients. The single low molecular mismatch risk patient that developed de novo DSA had two undetectable tacrolimus troughs over a period of 10 days prior to an episode of presumed cellular rejection at 8 weeks posttransplant, with subsequent DQ DSA detected on follow-up testing at 6 months with an MFI of 7000. The distribution of time-to-DR/DQ DSAs differed significantly by molecular mismatch risk category ($p < .0001$, Figure 1). In univariate analysis, there was a significantly higher risk of DR/DQ DSAs in intermediate (HR 13.92, 95% CI 1.85-104.94, $p < .001$) and high molecular mismatch risk (HR 21.64, 95% CI 2.94-159.38, $p < .001$) categories compared to the low molecular mismatch risk group (Table S3). Several traditional immunologic risk factors showed comparatively less robust associations with DR/DQ DSA development, including whole antigen DQ +DR mismatch of 3 – 4 compared to 1 – 2 (HR 2.58, 95% CI 1.34 – 4.97, $p = .005$), younger age (HR 0.97, 95% CI 0.95-0.99, $p = .002$), and deceased donor transplant (HR 2.4 95% CI 1.27-4.53, $p = .007$). Traditional risk factors that were not significantly associated with time-to-DR/DQ DSA development included ethnicity ($p = .13$), re-transplant ($p = .14$), delayed graft function ($p = .85$), and clinical acute cellular rejection ($p = .11$).

## 3.3 | Molecular mismatch and tacrolimus exposure

There were 9,092 trough levels analyzed before the development of DR/DQ DSA. Overall median number of tacrolimus trough measurements for all patients was 18 (IQR 13, 25) before de novo DR/DQ DSA or 12 months. Mean (± standard deviation, sd) tacrolimus trough over 12 months was 7.26 ± 1.31 ng/ml for the entire cohort and not statistically different between molecular mismatch risk groups ($p = .89$). Of the patients who had a mean tacrolimus trough <6.0 ng/ml, 16 (13%) were low molecular mismatch risk, 25 (16%) were intermediate risk, and 25 (15%) were high molecular mismatch risk ($p = 0.9$). In a multivariable model, patients with a mean tacrolimus trough <6.0 ng/ml in the first 12 months had an increased risk of DR/DQ de novo DSAs compared to those with a mean tacrolimus trough ≥8.0 ng/ml (HR 2.34 95% CI 1.05-5.22, $p = 0.04$; Table 3) while the hazard of DR/DQ de novo DSAs was over 15-fold (HR 15.39, 95% CI 2.01-118.09, $p = .009$) for intermediate versus low molecular mismatch risk and almost 24-fold (HR 23.81, 95% CI 3.17-178.66, $p = .002$) for high versus low molecular mismatch risk patients (Table 3). In a sensitivity analysis excluding the 11 recipients who developed dnDSA before the 6-month timepoint, intermediate (HR 12.71, 95%CI 1.6-99.6, $p = .016$) and high (HR 17.36, 95%CI 2.3-132.9,

$p$ = .006) HLA-DR/DQ molecular mismatch risk categories were still independently associated with higher risk of dnDSA development compared to low molecular mismatch risk after adjustment for other covariates. Comparable results were also found when the analysis was performed utilizing a positive/negative de novo DSA MFI threshold of ≥1000.

## 4 | DISCUSSION

The ability to precisely assess underlying immunologic risk of a given patient prior to transplant represents a critically important requirement to inform personalized patient management. Wiebe et al used HLA-DR$\beta_{1/3/4/5}$ and HLA-DQ$_{\alpha/\beta}$ molecule specific thresholds to predict intermediate and long-term DSA development in a single Canadian cohort.[9] Notably, the current study supported the same thresholds assigned in the Canadian cohort solely based on the analysis of the Denver cohort (Figure S1, Table S1 and S2). Furthermore, the same thresholds distributed patients similarly in both cohorts (low molecular mismatch risk: 27% vs. 25%, intermediate molecular mismatch risk: 35% vs. 36%, high molecular mismatch risk: 39% vs. 39%) regardless of different ethnicity profiles (eg, Hispanic and African American recipients) and allowed accurate prediction of DSA development despite differences in immunosuppression protocols and the posttransplant observation period (ie, <1 year posttransplant). HLA-DR/DQ molecular mismatch was able to identify a patient population that was at exceptionally low risk of developing DR/DQ DSAs despite traditional measures of alloimmune risk. In contrast, there was a graded increase in risk of DR/DQ DSAs in intermediate and high molecular mismatch risk patients, with over half of patients developing class II antibodies being in the high-risk group. These results validate the findings of Wiebe et al who also demonstrated a dramatically increased incidence of DSAs in intermediate and high molecular mismatch risk patients, with over 10-fold and 20-fold risk, respectively.

Standard tacrolimus maintenance dosing and dose reductions/escalation in response to clinical events can have profound implications on allograft survival and patient morbidity and mortality. Yet, there is no consensus on target tacrolimus troughs, with practical guidelines recommending a range of 5 – 10 ng/ml in the first year of transplant while others support tacrolimus minimization, targeting levels as low as 3 ng/ml.[11] This wide range in clinical practice is, in part, driven by a poor understanding of underlying immunologic risk. Gatault et al randomized 186 patients perceived as low immunologic risk at 4 months after transplant to a 50% reduction in tacrolimus with a target trough >3 ng/ml and the control group maintaining a target trough of 7 – 12 ng/m.[6] At 1 year, the low tacrolimus group had significantly more subclinical inflammation, clinical acute rejection, and de novo DSAs. Immunologic risk was defined by demographic and clinical variables without knowledge of the more specific HLA molecular mismatch. In the NIAID Clinical Trials in Organ Transplantation (CTOT)-09 study, "immune-quiescent" patients were randomized to tacrolimus withdrawal that culminated in alloimmune injury in the majority of patients and cessation of the trial.[12] Retrospectively, HLA-DQ eplet mismatch was determined. Despite the small number of recipients who had tacrolimus reduced or discontinued, HLA-DQ eplet mismatch was a statistically significant correlate of HLA-DQ de novo DSA development.

In the present study, reduced tacrolimus exposure had little impact on DSA development in patients with low molecular mismatch risk despite traditional measures of immunologic risk, including age, ethnicity, and type of donor. Wiebe et al evaluated tacrolimus exposure and DSA development in their cohort and found a similar relationship.[5] Low molecular mismatch risk patients who had a significantly higher percentage of tacrolimus levels below 5 ng/ml had similar risk of antibody development compared to patients above 5 ng/ml. In contrast, recipients with high eplet mismatch load were less likely to tolerate low tacrolimus levels without developing de novo DSAs. Similarly, higher tacrolimus exposure in the Denver cohort was associated with less DSA development in all molecular mismatch risk categories, with the low molecular mismatch group having only one patient with de novo DSA in the setting of documented nonadherence and undetectable tacrolimus troughs. HLA-DR/DQ molecular mismatch may be a more precise method to define immunologically low-risk patients who might tolerate lower drug exposure and may be an important tool that informs future clinical trial design.

T cell–mediated rejection can have an impact on allograft survival independent of antibody development.[13,14] Although HLA eplet mismatching has primarily been explored in relation to DSAs, it stands to reason that the degree of differences between self and non-self peptides on the HLA molecule increase the risk of T cell–mediated rejection, as well. We did not have sufficient histologic data to fully evaluate T cell–mediated rejection in this cohort. However, Wiebe et al found the same molecular mismatch categories used in this study significantly correlated with Banff borderline, Banff ≥IA, and Banff ≥IB T cell–mediated rejection free survival, providing further evidence for their alloimmune basis.[15] Additionally, the number of recurrent T cell–mediated rejections increased significantly with each grade of risk category.

This study has several notable limitations. Despite similar results in two independent cohorts, risk quantification should be interpreted with caution due to the small sample size and associated risk of type II error. Moreover, only 9% of patients in this study were African American, which is consistent with the Denver population but considerably less compared to 21% of all patients transplanted in the United States in 2019. The retrospective nature of this study introduces the possibility of important confounders that may have influenced tacrolimus dosing and risk of DSA development. Moreover, the small number of patients in the low-risk group who developed DSA supported our hypothesis but limited our ability to assess the interaction between tacrolimus exposure and the risk of class II de novo DSA in these patients. Patients were screened with LABScreen Mixed Beads

and confirmed with LABScreen Single Antigen Beads. Although no published reports directly compare the sensitivity of these assays, it is possible additional antibodies may have been detected by screening with single antigen beads. Finally, while class II de novo DSA is more commonly implicated in intermediate and late antibody-mediated injury, class I DSA development may also have pathologic sequela.[16–18]

In summary, this is the first study to validate that single molecule HLA molecular mismatch represents a reproducible, objective, and clinically relevant biomarker to stratify patients by alloimmune risk. Remarkably, this was demonstrated in a cohort with major demographic and clinical differences from the original cohort. These findings have wide-ranging potential to influence practice across the field of transplantation including the guidance of personalized immunosuppression management, living donor selection in kidney paired exchange programs, and for enrichment or stratification in clinical trial design. Prospective studies will be critically important to further define the role of molecular mismatching in organ transplantation.

## DISCLOSURE

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

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## DATA AVAILABILITY STATEMENT

Research data are not shared.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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