









ORIGINAL ARTICLE

Clinical importance of extended second field high-resolution HLA genotyping for kidney transplantation

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The need for extended second field high-resolution (2F-HR) HLA genotyping in kidney transplantation is debated. In a cohort of 1000 kidney transplants, we evaluated the impact of different HLA genotyping levels on the assignment of donor-specific anti-HLA antibodies (DSA) and investigated whether inference of 2F-HR genotypes from low-resolution (LR) genotypes could be used to correctly assign DSA. Based on LR genotypes, 224 pretransplant DSAs were present in 140 patients and absent in 860 patients (DSA_{neg} group). With extended 2F-HR HLA genotyping, we confirmed 173 DSA (77.2%) in 108 (77.1%) patients (2F-HR_{pos}LR_{pos} DSA group) and excluded DSA in 32 patients (22.9%) (2F-HR_{neg}LR_{pos} DSA group). Kaplan-Meier curves showed that 10-year graft survival rates were similar between the DSA_{neg} and 2F-HR_{neg}LR_{pos} DSA groups (82.4% vs 93.8%; $P = .27$) and confirmed that DSA determined using LR typing but not confirmed using 2F-HR typing were indeed misclassified. By inferring 2F-HR genotypes using *HaploStats*, DSA still could not be correctly assigned in 23.3% of cases. We conclude that extended 2F-HR typing of the donor-recipient pairs is relevant for the correct assessment of DSA. Although inference of 2F-HR genotypes may improve the assessment of DSA in some cases, significant misclassification occurs, and warrants caution in using inferred HLA results for clinical and research purposes.

KEYWORDS

basic (laboratory) research/science, clinical decision-making, clinical research/practice, histocompatibility, kidney transplantation/nephrology, major histocompatibility complex (MHC), organ procurement and allocation, panel reactive antibody (PRA), rejection: antibody-mediated (ABMR), risk assessment/risk stratification

1 | INTRODUCTION

Human leukocyte antigen (HLA) mismatches influence long-term graft outcome,¹ by the associated risk of T cell-mediated rejection.²

Moreover, the presence of antibodies that react with the mismatched donor's HLA type cause antibody-mediated rejection (ABMR) and premature graft failure.³⁻⁵ Therefore, in most transplant organizations, an HLA matching strategy was introduced. Organ allocation

Abbreviations: ABMR, histological phenotype of antibody-mediated rejection; DSA, donor-specific anti-HLA antibodies; ENIS, Eurotransplant Network Information System; HLA, human leukocyte antigen; HR, high-resolution; LR, low-resolution; MFI, median fluorescence intensity; NGS, next-generation sequencing; 2F-HR, second field high-resolution; SAB, single antigen bead.

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is still based on partial HLA-A,-B, and -DR antigen matching, and on the evaluation of anti-HLA antibodies against the mismatched donor's HLA type, defined at the low-resolution (LR) HLA antigen level.

For decades, the complement-dependent cytotoxicity test was the main test used to investigate the antibody reactivity pattern of the patient serum and to define unacceptable HLA mismatches. Currently, however, HLA laboratories use more sensitive solid-phase single antigen bead (SAB) assays with precise determination of HLA antibody specificities, at the second field high-resolution (2F-HR) HLA level.⁶ These antibodies sometimes react only with 1 allele that encodes 1 HLA molecule, or with a set of alleles that encode the same HLA molecule (2nd field HLA resolution). However, for allocation purposes, these reactive 2F-HR detected antibodies are converted into corresponding LR antigens and recorded in registries as unacceptable HLA antigens. SAB panels often have 2 or more 2F-HR HLA variants corresponding to the same HLA antigen. When not all 2F-HR HLA variants of a certain antigen share the same reactivity, this creates a dilemma in the interpretation of HLA compatibility at the antigen level, in the determination of unacceptable antigens and in the decisions on organ allocation.

As the number of unacceptable HLA antigens increases, the patient's chance to receive an organ offer decreases. To avoid this and to be more specific on the definition of unacceptable HLA mismatches, the United Network for Organ Sharing registry has the possibility to report unacceptable 2F-HR HLA variants instead of unacceptable HLA antigens. However, donor genotyping is currently still reported at the LR antigen level, which calls into question the real patient benefit of this strategy. Because no standard strategy is currently available for the exclusion of 2F-HR unacceptable HLA variants, each transplant center decides whether an antigen mismatch is unacceptable or not when second field HLA-specific antibodies are detected.⁷

Donor HLA genotyping at the 2F-HR HLA level could resolve these clinical dilemmas and more accurately assess the HLA compatibility of transplant pairs. Although technologies, like RealTime PCR or the most powerful next-generation sequencing (NGS), offer the possibility to genotype the transplant pair at the 2F-HR HLA level for 11 HLA loci, its clinical usefulness is still a matter of debate within the transplant community.^{8,9} To date, no studies have assessed the added clinical value of 2F-HR extended HLA typing over LR HLA-A,-B, -DR, and -DQ typing in solid organ transplantation, and the LR genotyping techniques remain the cornerstone for determining the HLA compatibility between donor-recipient pairs.

To better risk-stratify patients on their likelihood of experiencing immune-mediated injuries and guiding personalized immunosuppression, investigating the eplet incompatibility is gaining momentum in the field of kidney transplantation. Also, the eplet analysis requires 2F-HR genotyping data, but many studies use statistical approaches to obtain untyped HLA data. Two different concepts exist to estimate untyped HLA data based on the linkage disequilibrium between HLA alleles of different loci. (1) Imputation tools estimate the missing untyped genotypes from neighboring typed single nucleotide polymorphisms using a reference panel. These imputation tools have become the primary tools used in case-control association

studies to search for genetic determinants of complex diseases.¹⁰ (2) Inference tools infer the 2F-HR genotypes from the LR results based on the observed HLA allelic frequencies and haplotypes in different ethnic populations and are often used in the transplant field.¹¹⁻¹⁴ The possibility to estimate the 2F-HR HLA data brings additional confusion in the discussion about the clinical value of 2F-HR genotyping methods in the field of solid organ transplantation.¹⁵ Although it is possible to obtain the 2F-HR genotypes correctly from the LR genotypes for some individuals,¹⁶ it remains unknown whether such inference affects calculations of donor-recipient eplet incompatibility.

Therefore, we aimed to evaluate the clinical utility of LR vs 2F-HR HLA genotyping on the assignment of donor-specific anti-HLA antibodies (DSA) from SAB test results. Additionally, we investigated whether the inference of the donor 2F-HR genotypes from the LR genotyping data could be used to correctly assign the presence of DSA and accurately estimate the eplet mismatch repertoire.

2 | METHODS

2.1 | Patients

All consecutive adult single kidney transplantations performed at the University Hospitals-Leuven between March 1, 2004 and February 6, 2013 were eligible for this study (N = 1000). All transplantations were ABO-compatible and were performed with negative complement-dependent cytotoxicity crossmatches on T and B lymphocytes. No patient received preconditioning HLA antibody desensitization. All clinical data were prospectively collected during routine clinical follow-up. This study was approved by the Ethics Committee of the University Hospitals Leuven (S53364 and S61788).

2.2 | HLA genotyping data

Recipient and donor LR HLA genotypes for HLA-A,-B and -DR were obtained from the Eurotransplant Network Information System (ENIS), at split antigen level. Available donor and recipient DNA samples, prospectively collected in the Biobank Renal Transplantation of the University Hospitals Leuven, were retrospectively genotyped at the LR and 2F-HR levels. For patients with HLA-C,-DQB₁, and/or DP antibodies, the donors were further genotyped for HLA-C,-DQB₁ or -DPB₁ if the typing data were not available in ENIS. Molecular typing was performed for C and DQB₁ loci by polymerase chain reaction (PCR) amplification with sequence-specific primers (Olerup SSP) or for DPB₁ (exon2) by Sanger sequencing (SBT, Protrans GmbH). We used these data to assign DSA at the LR level. In addition, all recipients (N = 1000) and donors (N = 932) with available DNA samples were genotyped retrospectively at the 2F-HR level for HLA-A,-B,-C,-DRB₁,-DRB₃,-DRB₄,-DRB₅,-DQA₁,-DQB₁,-DPA₁, and -DPB₁ loci using NGS. Donors were genotyped using the commercially available MIA FORA NGS FLEX 11 HLA Typing Kit (Immucor) on

the MiSeq sequencing instrument (Illumina Inc) or using the HiSeq sequencing system for typing exon 2, 3, and 4 for HLA -A,-B,-C and exon 2 and 3 for -DRB₁,-DRB₃,-DRB₄,-DRB₅,-DQA₁,-DQB₁,-DPA₁, and -DPB₁. The latter was also used to retype all recipients. These NGS genotyping results were used to define DSA at the 2F-HR HLA level. The 2F-HR HLA typing level discriminates all the coding differences of the extracellular HLA domains between the HLA alleles. These extracellular HLA domains define a unique HLA protein composition presented on the cell membrane.

2.3 | Methods for inferring 2F-HR HLA results

We evaluated 2 different methods to infer the 2F-HR HLA results of the donors. Using the HLAMatchmaker 4-digit allele converter program v1 (file name: 6ABCDRB1DQBconverterVs1), we obtained the 2F-HR genotypes for A,-B,-C,-DRB₁ and -DQB₁ loci from the LR data at split antigen level for HLA-A,-B,-DR.¹⁶ The HaploStats (www.haplostats.org) tool allowed us to estimate the 2F-HR results for HLA-A,-B,-C,-DRB₁,-DRB₃,-DRB₄,-DRB₅ and -DQB₁ from the LR HLA-A,-B,-C,-DRB₁ and -DQB₁ genotypes.¹⁷ In both methods, we selected the most likely 2F-HR HLA genotypes according to the highest haplotype frequency in European whites.

2.4 | Detection of circulating anti-HLA antibodies and assignment of DSA

Anti-HLA antibodies were systematically monitored in 1 histocompatibility laboratory (HLA-Red Cross-Flanders). In case this was not done in the clinical routine with the Luminex technology, we routinely retested bio-banked sera (n = 435 pretransplant day 0 samples) for the presence of HLA antibodies and circulating DSA in the same laboratory. All sera were first screened using a LIFECODES LifeScreen Deluxe kit (Immucor), and in case of positive screening, the donor specificity was assessed using LIFECODES SAB kits (Immucor). Ethylenediaminetetraacetic acid was added to serum (1:10) to avoid the prozone effect. The cut-off for the presence of circulating HLA antibodies and DSA was a median fluorescence intensity (MFI) value ≥ 500 if the background-corrected ratio or antigen density background-corrected ratio was ≥ 4 .

2.5 | Kidney allograft biopsies and histological scoring

We included all posttransplant renal allograft biopsies, performed up to 5 years after transplantation (N = 3647). Timing and scoring of the biopsies were described in detail previously.¹⁸ The diagnosis of the histological phenotype of ABMR_h was based on the first 2 criteria for active ABMR, as defined by the Banff 2017 consensus.¹⁹

2.6 | Statistical analysis

When comparing 2 groups, Pearson's χ^2 test was used for categorical data, the 2-sample t test for the continuous variables, and the Wilcoxon test for the comparison of medians. Survival curves were plotted using the Kaplan-Meier method and groups were compared by log-rank testing. In the case of death with a functioning graft, we censored graft survival at the time of death. The log-rank test

TABLE 1 General characteristics and follow-up of the study population (N = 1000)

Characteristics	Entire cohort (N = 1000)
Recipient characteristics at transplantation	
Age (y), mean \pm SD (range)	53.7 \pm 13.3 (16.2-82.6)
Recipient BMI at time of transplantation (kg/m ²), mean \pm SD (range)	25.4 \pm 4.5 (15.5-42.0)
Sex (male), n (%)	609 (60.9)
White, n (%)	984 (98.4)
Repeat transplantation, n (%)	154 (15.4)
Diabetes mellitus, n (%)	166 (16.6)
Donor characteristics at transplantation	
Age (y), mean \pm SD (range)	47.7 \pm 14.8 (5.2-79.0)
Sex (male), n (%)	535 (53.5)
Deceased donor, n (%)	941 (94.1)
Donation after brain death, n (%)	780 (78.0)
Transplant characteristics, treatment at transplantation and follow-up	
Cold ischemia time (h), mean \pm SD (range)	14.2 \pm 5.7 (0.17-36.0)
Immunosuppression regimen: TAC-MPA-CS, n (%)	874 (87.4)
Induction therapy, n (%)	416 (41.6)
Basiliximab, n (%)	355 (35.5)
Thymoglobulin, n (%)	16 (1.6)
Other, n (%)	45 (4.5)
Patients with anti-HLA antibodies, n (%)	262 (26.2)
HLA class I, n (%)	94 (9.4)
HLA class II, n (%)	60 (6.0)
HLA class I & II, n (%)	104 (10.4)
Patients with DSA at LR level, n (%)	140 (14.0)
Posttransplant data	
Follow-up time (y), median (IQR)	7.5 (4.9-10.0)
Death-censored graft survival	
At 1 y (%)	95.2
At 5 y (%)	89.1
At 10 y (%)	80.7

Abbreviations: BMI, body mass index; CS, corticosteroids; DSA, donor-specific anti-HLA antibodies; HLA, human leukocyte antigen; IQR, interquartile range; LR, low-resolution; MPA, mycophenolic acid; SD, standard deviation; TAC, tacrolimus.

3.3 | Comparison between HR_{neg}LR_{pos} DSA and HR_{pos}LR_{pos} DSA groups

To examine whether the misclassifications by using LR genotyping were clinically relevant, we analyzed the impact of the misclassified antibodies on long-term graft survival and graft histology. We categorized the patients in 3 groups: (1) no DSA (DSA_{neg}); (2) presence of only misclassified DSA (according to LR genotyping; 2F-HR_{neg}LR_{pos} DSA); and (3) the presence of at least 1 confirmed DSA by 2F-HR genotyping (2F-HR_{pos}LR_{pos} DSA). One hundred eight of 140 (77.1%) patients had at least 1 confirmed DSA by 2F-HR genotyping (2F-HR_{pos}LR_{pos} DSA group), and the remaining 32/140 (22.9%) patients had only misclassified DSA (2F-HR_{neg}LR_{pos} DSA group). Kaplan-Meier survival analysis showed that 10-year graft survival rates, censored for recipient death, were similar between the DSA_{neg} and 2F-HR_{neg}LR_{pos} DSA groups (82.4% vs 93.8%; $P = .27$ by log-rank test), which were both significantly better than graft survival in the 2F-HR_{pos}LR_{pos} DSA group (62.3%; $P < .0001$ and $P = .0007$, respectively) (Figure 3A). Also, when we investigated the histology data of posttransplant biopsies performed in this cohort, we found that none of the 32 2F-HR_{neg}LR_{pos} DSA patients

developed the histological phenotype of ABMR (ABMR_h) within the first 5 years during the biopsy follow-up (Figure 3B). Therefore, we could not identify an association between misclassified DSA at LR level and ABMR or graft survival, confirming that these antibodies were also misclassified from the clinical perspective.

Next, by comparing the HLA mismatches on LR and 2F-HR levels, the overall HLA mismatches differed between the 2F-HR_{neg}LR_{pos} DSA and 2F-HR_{pos}LR_{pos} DSA group (Table 2). In terms of the DSA, although the MFI values were not statistically different, there was a higher percentage of DSA ≥ 2000 MFI in the 2F-HR_{pos}LR_{pos} DSA compared with the 2F-HR_{neg}LR_{pos} DSA group (59.5% vs 42.5%, $P = .05$).

3.4 | Impact of inferred 2F-HR HLA genotypes on DSA assignment

We then used HLA-Matchmaker and HaploStats to infer the donor 2F-HR HLA genotypes from the LR_{pos} DSA group ($N = 134$). Six transplant pairs of this group did not have sufficient DNA material and not all 11 loci could be genotyped at the 2F-HR level. With HLA-Matchmaker, donors' 2F-HR genotypes were correctly obtained in 87.9%, 71.5%, 50.9%, 49.5%, and 57.8% for HLA-A, -B, -C, -DRB₁, and -DQB₁ loci, respectively (Figure 4A). By using the Haplostat tool, donors' 2F-HR genotypes were correctly obtained in 97.5%, 90.0%, 89.6%, 83.3%, 70.7%, and 84.0% for HLA-A, -B, -C, -DRB₁, -DRB₃₄₅ and -DQB₁ loci, respectively (Figure 4B). Because of the higher accuracy of the HaploStats tool, we used only these inferred high-resolution (HR) donors' genotypes in further analysis.

Using the Haplostats-inferred donor 2F-HR HLA genotypes of transplants with LR misclassified DSA (the 2F-HR_{neg}LR_{pos} DSA group), DSA could be correctly excluded in 18/21 (85.7%) of the patients who required 2F-HR genotyping results, thus were wrongly classified in 3/21 (14.3%) cases. In the remaining 11 patients, DSA could not be excluded as DQA₁, DPB₁ and DPA₁ loci could not be inferred by Haplostats. However, when we used the inferred donors' HR HLA genotypes to reassess the presence of DSA in the 2F-HR_{pos}LR_{pos} DSA group, we found that DSA would be wrongly excluded in 20 (18.5%) patients (2F-HR_{pos}Inferred_{neg} DSA) and correctly confirmed in 88 (81.5%) of patients (2F-HR_{pos}Inferred_{pos} DSA). We confirmed the clinical implications of DSA misclassification based on the inference of 2F-HR HLA genotyping in survival analysis. Patients with wrongly excluded DSA (2F-HR_{pos}Inferred_{neg} DSA) had the lowest 10-year graft survival rate of 44.9% and the highest cumulative incidence of ABMR_h (91.2%) within the first 5 years posttransplant (Figure 5).

From this, we evaluated the accuracy of the 3 different donor HLA genotyping levels for assigning DSA in HLA-sensitized patients ($N = 262$). Using the 2F-HR genotyping results as the criterion standard for comparison to the LR and inferred 2F-HR genotyping, the specificity was higher for the inferred donor typing data (89.6%) than for the LR donor data (81.8%). However, the accuracy of LR genotyping was 79.8%, even better than the accuracy of 76.7% for the inferred 2F-HR donor data due to the lower sensitivity of the inferred genotypes (Table 3).

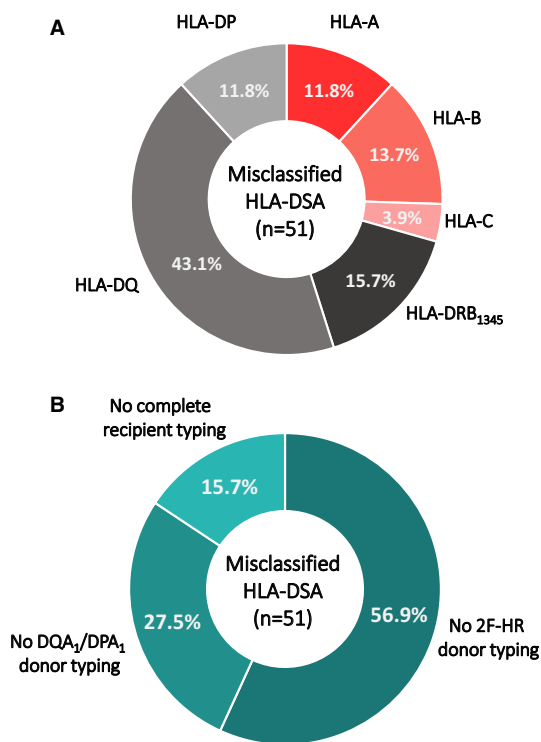


FIGURE 2 Distribution per HLA molecule/locus and reasons for DSA misclassification based on LR genotyping ($n = 51$). A, The majority of misclassified DSA were directed against class II (70.6%), especially against the DQ molecule (43.1%). B, The reasons for DSA misclassification were the following: not typing the donor at the second field HR level (56.9%); not typing DQA₁/DPA₁ loci of the donor (27.5%); and incomplete recipient typing at the second field HLA level (15.7%). 2F-HR, second field high-resolution; DSA, donor-specific anti-HLA antibodies; HLA, human leukocyte antigen; LR, low-resolution genotyping level [Color figure can be viewed at wileyonlinelibrary.com]

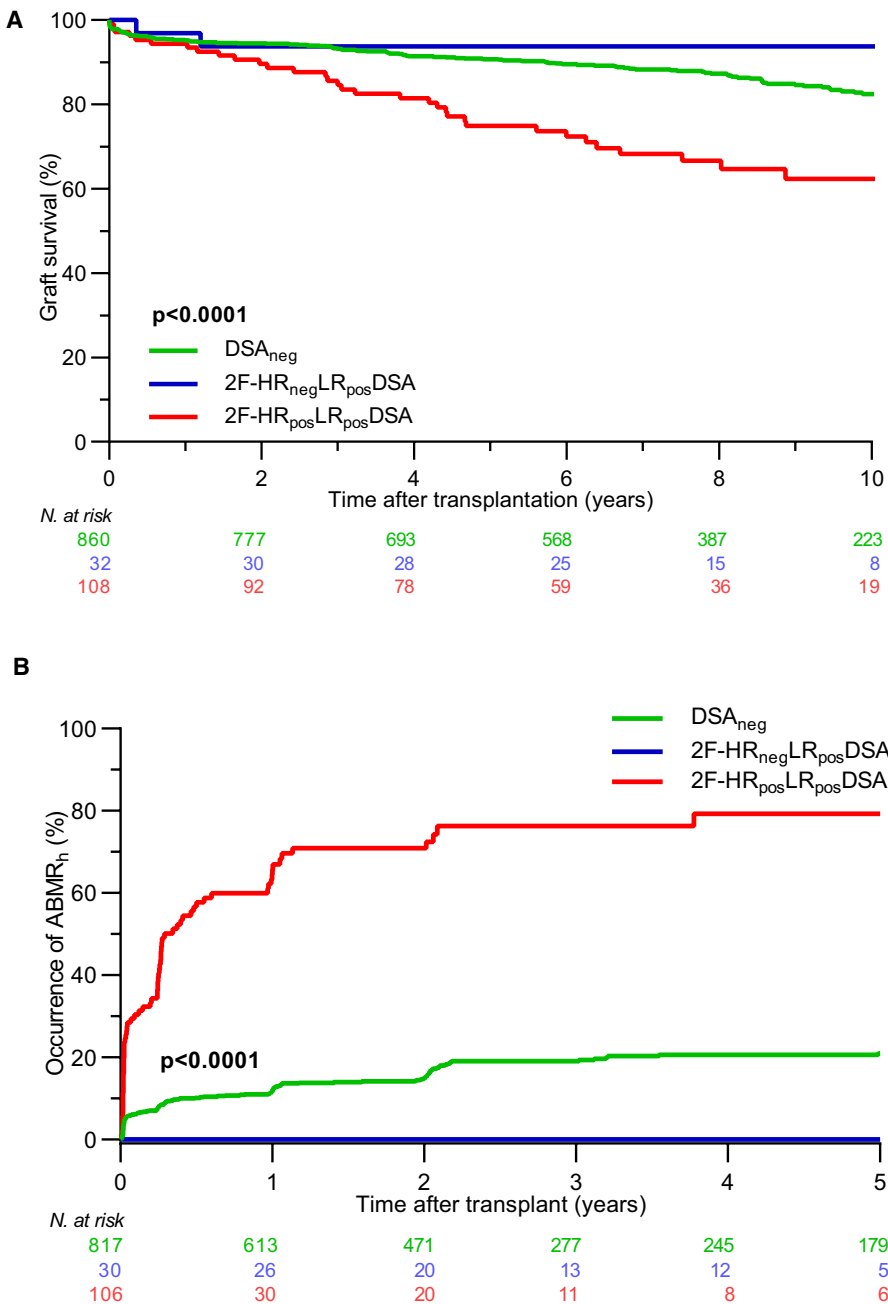


FIGURE 3 Survival analysis and occurrence of ABMR_h, stratified according to the presence of DSA determined by LR vs HR genotyping. A, Ten-year death-censored graft survival was inferior only for patients with confirmed DSA by HR genotypes (2F-HR_{pos}LR_{pos}DSA) when compared to patients with DSA defined only by LR and not confirmed by HR typing (2F-HR_{neg}LR_{pos}DSA), and when compared to DSA-negative patients (62.3% vs 93.8% vs 82.4%, $P < .0001$ by log-rank test). B, The cumulative incidence of ABMR_h within the first 5 years was 21.0%, 0.0%, and 79.3% for DSA_{neg}, 2F-HR_{pos}LR_{pos}DSA, and 2F-HR_{neg}LR_{pos}DSA group, respectively. Forty-seven patients without biopsy follow-up were excluded from this analysis. ABMR_h, histological phenotype of antibody-mediated rejection; DSA, donor-specific anti-HLA antibodies; 2F-HR, second field high-resolution; LR, low-resolution; neg, negative; pos, positive [Color figure can be viewed at wileyonlinelibrary.com]

3.5 | Impact of the inferred or incomplete HLA type on the donor-recipient eplet incompatibility

Finally, we investigated the impact of the inferred 2F-HR donor genotypes on the eplet mismatch load repertoire in the patients with available LR HLA-A,-B,-C,-DR and -DQ donor typing data ($N = 471$). By using the *HaploStats* tool, we obtained inferred 2F-HR results for 469 donors. In this cohort, donors' 2F-HR genotypes were correctly estimated in 96.3%, 91.3%, 95.4%, 81.7%, 67.3%, and 81.5% for HLA-A,-B,-C,-DRB₁,-DRB_{3/4/5} and -DQB₁ loci, respectively. First, we evaluated the impact of using inferred donors' genotypes in the assessment of eplet mismatch load. For this, we calculated differences in the eplet(s) between the eplet load of the actual 2F-HR genotypes and the eplet

load of the inferred donors 2F-HR genotypes for each transplant pair, for both class I and class II molecules ($N = 469$). The agreement in the total number of mismatched eplets was 91.3% for class I (A, B, and C) and 53.9% for class II (DRB_{1/3/4/5} and DQB₁) (Figure 6A). Considering the differences in the mismatched eplet repertoire instead of the total number of mismatched eplets, the agreement between the inferred and the real 2F-HR genotypes was 75.3% for class I and 35.4% for class II. This illustrates that the currently used methods for inferring second field HLA results do not allow correct evaluation of the eplet mismatch repertoire, especially of class II molecules.

Because the DQ molecule is currently extensively studied at the eplet mismatch level for risk stratification due to the high incidence of de novo DSA formation, several studies focus on DQB₁ data.¹¹⁻¹⁴

TABLE 2 Immunological profiles of patients with pretransplant DSA, according to the accuracy of the DSA estimated using LR genotyping (N = 140 individual transplantations and N = 224 different DSA)

Transplant characteristics	2F-HR _{pos} -LR _{pos} -DSA	2F-HR _{neg} -LR _{pos} -DSA	p value
	N = 108	N = 32	
HLA mismatches			
At split antigen HLA level			
HLA-A/B/DR mismatches (0-6), mean ± SD	2.9 ± 1.2	2.2 ± 1.3	.01
A antigen, mean ± SD	0.9 ± 0.7	0.7 ± 0.6	.06
B antigen, mean ± SD	1.1 ± 0.6	0.9 ± 0.7	.17
DR antigen, mean ± SD	0.8 ± 0.6	0.6 ± 0.6	.09
At 2F-HR HLA level ^a	103	31	
Total 2F-HR HLA mismatches (0-16), mean ± SD	8.7 ± 2.3	7.6 ± 2.6	.03
A locus, mean ± SD	1.7 ± 0.7	1.4 ± 0.8	.11
B locus, mean ± SD	1.3 ± 0.6	1.2 ± 0.8	.35
C locus, mean ± SD	1.4 ± 0.6	1.1 ± 0.8	.06
DRB ₁ locus, mean ± SD	1.2 ± 0.7	1.0 ± 0.7	.37
DQB ₁ locus, mean ± SD	1.0 ± 0.6	0.8 ± 0.5	.11
DQA ₁ locus, mean ± SD	0.9 ± 0.6	0.9 ± 0.6	.80
DPB ₁ locus, mean ± SD	1.1 ± 0.7	1.0 ± 0.7	.71
DPA ₁ locus, mean ± SD	0.3 ± 0.6	0.2 ± 0.4	.39
DSA characteristics^b			
Total number of DSA at LR level ≥500 MFI	184	40	
Patients with multiple DSAs, n (%)	53 (49.1)	7 (21.9)	.006
Misclassified DSA compared to 2F-HR, n	11	40	
Total number of assigned DSA per group	173	40	
DSA class II, n (%)	93 (53.8)	26 (65.0)	.20
DSA ≥ 1000 MFI, n (%)	144 (83.2)	31 (77.5)	.39
DSA ≥ 2000 MFI, n (%)	103 (59.5)	17 (42.5)	.05
MFI value of DSA, median (IQR)	2593 (1328-5146)	1507 (1039-3678)	.08

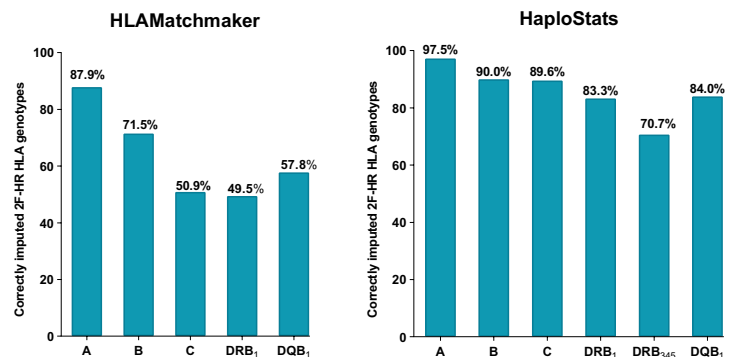
Significant differences are highlighted in bold.

Abbreviations: 2F-HR, second field high-resolution; DSA, donor-specific HLA antibodies; HLA, human leukocyte antigens; HR, high-resolution; IQR, interquartile range; LR, low-resolution; MFI, median fluorescence intensity; SD, standard deviation.

^aComplete 2F-HR HLA typing was not available for 6 transplant pairs.

^bSome of the patients have multiple DSA.

FIGURE 4 Accuracy of inferred 2F-HR HLA typing results using *HaploStats* (A,-B,-C,-DR,-DQ input) and *HLAMatchmaker* (A,-B,-DR input) programs in the LR_{pos}-DSA cohort with available full 2F-HR genotypes (N = 134). Bars on the graph represent the percentage of correctly inferred second field HLA genotypes per locus. DSA, donor-specific anti-HLA antibodies; 2F-HR, second field high-resolution; LR, low-resolution; HLA, human leukocyte antigen; pos, positive [Color figure can be viewed at wileyonlinelibrary.com]



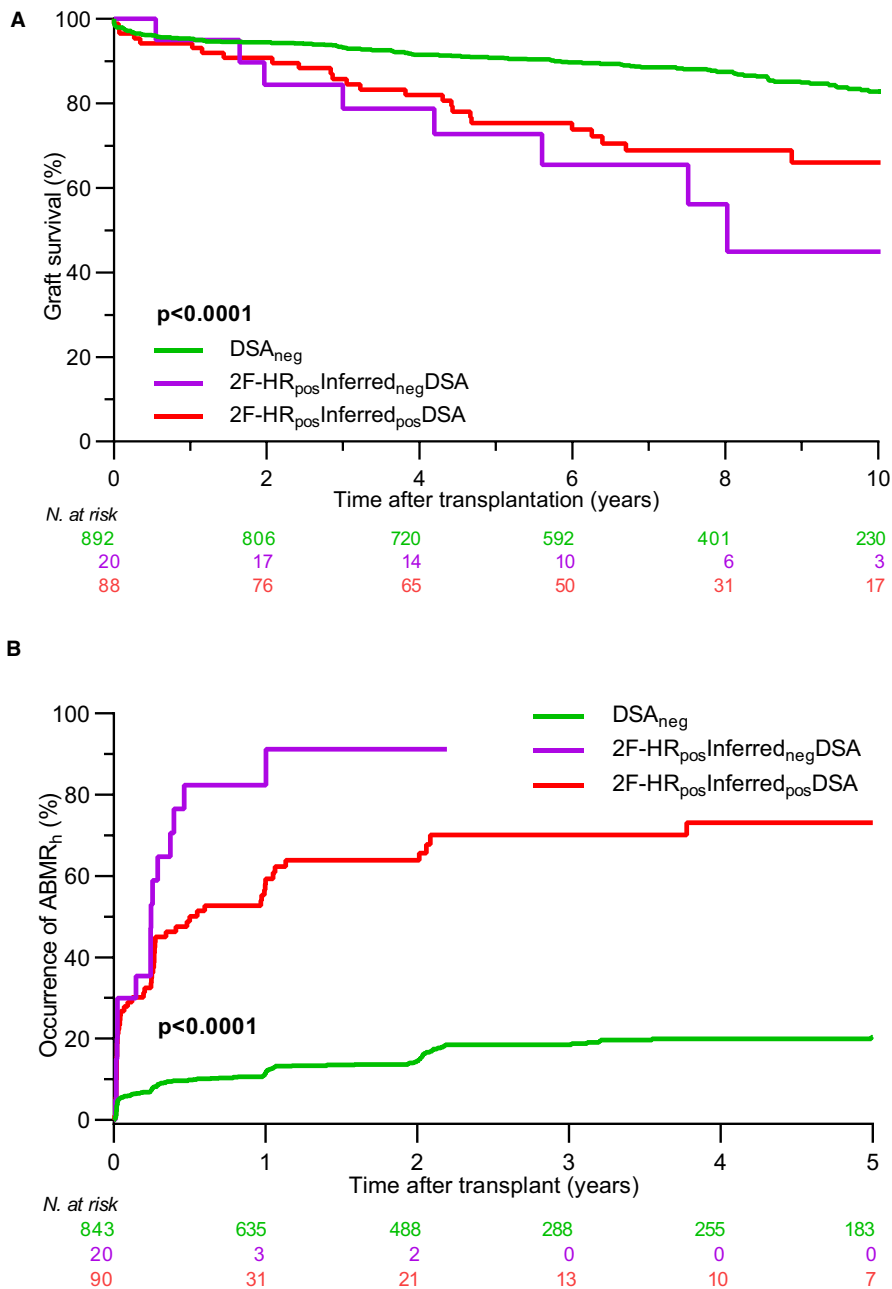


FIGURE 5 Survival analysis and occurrence of $ABMR_h$, stratified according to the presence of DSA assessed by inferred vs true 2F-HR genotypes. A, Both the $2F-HR_{pos} Inferred_{pos} DSA$ group and the $2F-HR_{pos} Inferred_{neg} DSA$ group had inferior 10-year death-censored graft survival compared to DSA_{neg} patients (66.0% vs 44.9% vs 82.8%, $P < .0001$ by log-rank test). B, The cumulative incidence of $ABMR_h$ within the first 5 years was 20.4%, 73.1%, and 91.2% for DSA_{neg} , $2F-HR_{pos} Inferred_{pos} DSA$, and $2F-HR_{pos} Inferred_{neg} DSA$, respectively ($P < .0001$ by log-rank test). Forty-seven patients without biopsy follow-up were excluded from this analysis. $ABMR_h$, the histological phenotype of antibody-mediated rejection; DSA, donor-specific anti-HLA antibodies; 2F-HR, second field high-resolution; neg, negative; pos, positive [Color figure can be viewed at wileyonlinelibrary.com]

Therefore, we evaluated the differences in the mismatch eplet repertoires when the whole DQA_1B_1 molecule is assessed compared to the DQB_1 only. We found that only in 37.8% of the cases, the eplet mismatch load is the same between the whole DQA_1B_1 molecule and the DQB_1 only, while in 62.2% the eplet repertoire in the eplet mismatch load differs from 1 to 17 eplets (Figure 6B).

4 | DISCUSSION

In the current study, we investigated how 2F-HR HLA genotyping impacts the characterization of HLA antibodies detected by solid-phase SAB, and whether inference of the 2F-HR genotypes from the LR genotypes could be used to correctly assign the presence

of DSA and accurately estimate the eplet mismatch repertoire. We showed that in one-fifth of the pretransplant DSA suspected by LR HLA genotypes, the donor specificity could not be confirmed after 2F-HR genotyping, primarily related to misclassifications of HLA-DQ antibodies. We also showed that although inference of the 2F-HR genotypes from LR data improved the assessment of DSA of misclassified cases, significant misclassifications still occurred in $\approx 20\%$, calling into question the relevance of such inference for clinical purposes. The accuracy for assigning DSA in sensitized patients was 79.8% for LR genotyping and 76.7% for inferred 2F-HR donor HLA genotyping using HaploStats. Finally, due to inaccuracies in the eplet compatibility estimation, mainly for HLA class II, complete 2F-HR HLA genotype measurement is recommended for clinical and research purposes.

TABLE 3 Comparison of the accuracy of DSA assignment with LR and inferred 2F-HR genotyping with HaploStats. Donor-recipient 2F-HR HLA genotyping was considered the criterion standard (N = 262 patients with anti-HLA antibodies)

	Donor genotyping HLA level			
	LR split antigen level		Inferred 2F-HR level	
DSA \geq 500	pos	neg	pos	neg
Final DSA positive	83	25	63	45
Final DSA negative	28	126	16	138
Accuracy (%)	79.8		76.7	
Sensitivity (%)	76.9		58.3	
Specificity (%)	81.8		89.6	
PPV (%)	74.8		79.8	
NPV (%)	83.4		75.4	

Abbreviations: 2F-HR, second field high-resolution; DSA, donor-specific anti-HLA antibodies; HLA, human leukocyte antigens; LR, low-resolution; neg, negative; NPV, negative predictive value; pos, positive; PPV, positive predictive value.

Analyzing the patients' sera reactivity of the HLA antibodies detected by solid-phase SAB is a challenging task when the traditional LR donor genotyping approaches are applied. Our finding that one-fifth of the cases with DSA were misclassified when using LR donor genotypes has important clinical consequences. Patients with DSA at LR level that are disproved by 2F-HR typing could be wrongly classified as having high immunological risk and receive more aggressive immunosuppression and specific antibody-targeting therapies, while the outcome is good in such cases even without therapeutic intervention. Although inferring the 2F-HR genotypes can improve the assessment of DSA in these patients, by excluding a relevant percent of misclassified DSA at LR level, clinically significant misclassification remains in the total DSA cohort. Our data thus suggest that HLA compatibility and mismatch acceptability need to be determined at the 2F-HR level, in order to allow correct assessment of the donor-specificity of the HLA antibodies. These findings echo the results of a recent case series, where 2F-HR HLA genotypes, obtained from routine clinical practice, helped in the correct assessment of the presence of DSA.²⁰

Our findings also have important implications for the allocation of kidneys in HLA-sensitized patients. We found that one-fifth of the patients with DSA assessed at the antigen level do not have a higher risk of rejection and graft failure than the DSA-negative patients. Suitable organs at the 2F-HR level might be declined for these patients due to the wrongly assumed high immunological risk evaluated at the antigen level. By better characterization of the donor genotypes at the 2F-HR HLA level, and thus ruling out any DSA misclassification, the chances of receiving a transplant offer would not decrease as one could fear.⁹ On the contrary, by not excluding the whole HLA antigen groups, the donor pool for finding compatible donors will increase. Finally, having second field HLA variants available for donor-recipient pairs would also optimize and unify the listing of unacceptables for the sensitized renal transplant candidates, and would allow correct assessment of the broadness of HLA sensitization as quantified by panel reactive antibodies.²¹

In our cohort, HLA-DQ was the molecule most needing 2F-HR genotyping for correct evaluation. Of all misclassified DSAs at the LR level, 43% were directed against the DQ molecule. There were 3 main issues that complicated the correct interpretation of the DQ antibody reactivity based on LR genotyping: (1) insufficient LR genotyping level of the donor DQB₁ locus, which level does not allow accurate determination of the DQB₁ reactivity patterns; (2) absence of genotyping information for DQA₁ locus, and therefore wrong attribution of anti-DQA₁ reactivity to DQB₁ in some cases;²² and (3) inability to analyze the sera reactivity toward the physiological donors' DQ molecules as not all DQA₁/DQB₁ donor dimers are present in the SAB assays. Because the DQ molecule is associated with the highest incidence of de novo DSA occurrence and poor graft outcome, further studying of DQ immunogenicity at the molecular level should be the main focus of research.²³

Due to the high inaccuracy of the eplet mismatch repertoire, especially for HLA class II, our results warrant caution in the interpretation of studies that use inferred HLA genotypes, as was also concluded in another recent study.²⁴ HLA genotype inference may impact the conclusions of such studies by influencing the accuracy of the DSA evaluation, and also by including substantial inaccuracies in the eplet mismatch load calculation. When studying molecular HLA mismatches between organ donors and recipients, genotyping at the 2F-HR level seems crucial, especially for the DQ locus.

It may be argued that the threshold used for assigning DSA positivity in our cohort of 500 background-corrected MFI value is low. Although this may well be true for some cases of misclassified DSA, it should be noted that increasing the MFI threshold could lead to neglecting pathogenic DSAs due to the known phenomenon of artificial reduction of the MFI in the SAB assay.²⁵ This phenomenon is caused by a common epitope that is recognized by a single antibody but present on multiple beads in the SAB assay. Therefore the measured MFI value of a single bead sometimes does not always accurately represent the titer of a specific anti-HLA antibody. For this, the complete donor 2F-HR genotyping data can help in the data interpretation by identifying common epitopes or amino acid residues

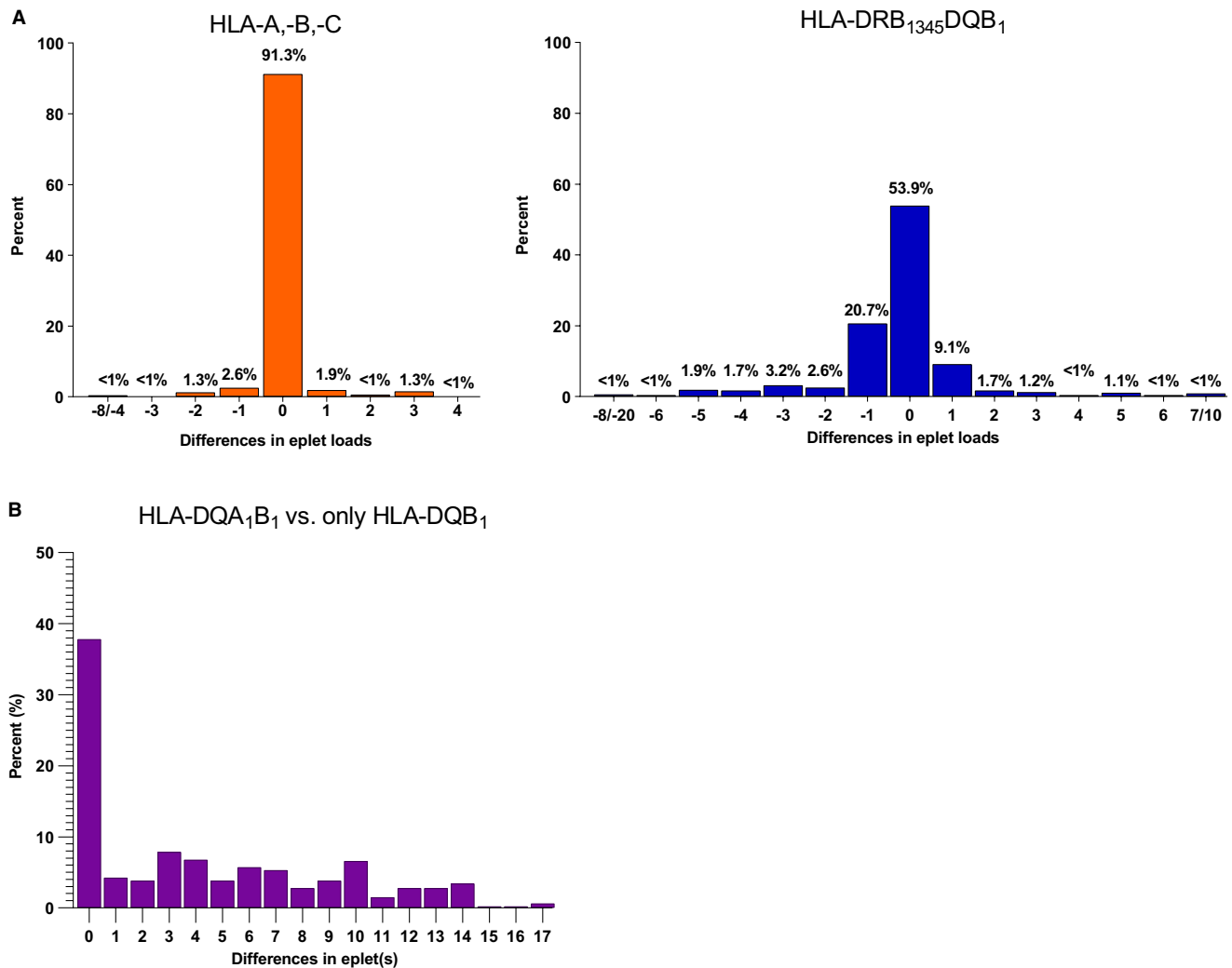


FIGURE 6 Differences in the eplet mismatch repertoire when using incomplete or inferred donor HLA genotypes. The differences are calculated in eplet(s) and the x-axis shows the difference in eplets between the eplet mismatch loads. A, Differences in the eplet mismatch loads between true vs inferred HLA genotypes for HLA-A/-B/-C and DRB₁₃₄₅/DQB₁. The differences refer only to the mismatch load, not to the eplet mismatch repertoire. Considering the differences in the mismatched eplet repertoire instead of the total number of mismatched eplets, the agreement between the inferred and the real 2F-HR genotypes was 75.3% for class I and 35.4% for class II. B, Differences in the eplet mismatch loads between the eplet load of the complete DQ (DQA₁/DQB₁) molecule vs the eplet mismatch load calculated only for DQB₁ locus. 2F-HR, second field high-resolution; HLA, human leukocyte antigen [Color figure can be viewed at wileyonlinelibrary.com]

on different beads that are involved in antibody reactivity patterns. Also, the complete recipient 2F-HR genotyping, used as additional locus-specific negative controls, helps excluding unspecific reactivities when going above the cutoff for DSA positivity in the final DSA, as was the case in 15.7% of the misclassified DSAs in our study. This also provides an opportunity to avoid the use of fixed cutoff MFI values for DSA positivity and to determine more specifically the anti-HLA sera reactivity.

Our study has several limitations. First, we do not have data on the donor ethnicity, which may have impacted the inference of the 2F-HR HLA. However, as the Eurotransplant allocation policy does not allow the recording of donor ethnicity, this HLA inference accuracy reflects the accuracy that would be achieved if HLA inference were used for clinical purposes. Also, we did not test the second vendor of SAB tests to investigate the correct donor molecules

that were not present in our reagents. In these few cases, we analyzed the sera reactivity towards the HLA molecules present in the SAB assay that have the same sequences in the extracellular domains (exons 2, 3, 4, for class I and exons 2, 3 for class II) as the donor molecule. This was the case mainly for the HLA-DP molecule. Therefore, the possibility of missed DSA in the DSA-negative group is very low. Also, for a few patients, the donors' DQA₁B₁ dimers were not present in our LSA2 kit. Therefore, we assigned DSA only against DQA₁ or DQB₁ molecules. It can be anticipated that the lack of the correct donor dimer can underestimate the real strength of the DSA. Finally, we did not apply specific antibody-targeting desensitization therapies in this population. While this allowed evaluation of the natural presentation of the DSA-positive patients, outcome data could be different in more recent years with the advent of desensitization options for patients with pretransplant DSA.

Re-evaluation of our conclusions in such recent cohorts is therefore necessary.

In summary, our study provides clinical support for extended 2F-HR HLA genotyping of transplant pairs to correctly determine the presence of DSA. Extended 2F-HR HLA genotyping also seems relevant in more accurately defining the HLA compatibility in a given donor/recipient combination, to eventually adopt preventative strategies for allograft rejection, to guide follow-up intensity, and to improve decisions on DSA-targeting therapies. Therefore, for all living donor kidney transplantations in sensitized recipients, NGS (or another methodology able to provide 2F-HR typing) should be done. The currently available NGS methods (or other methods for 2F-HR typing) do not meet the turnaround time needed for deceased donor typing. The real-time quantitative PCR or HR sequence-specific oligonucleotide methods should be used for rapid deceased donor typing as they increase the number of targeted HLA loci and discriminate most of the common second field HLA variants present in the SAB kits. If the 2F-HR typing level cannot be achieved in the pre-transplant setting, donor-retyping with NGS (or another 2F-HR typing method) needs to be performed as soon as possible post-transplant in sensitized recipients. Such posttransplant retyping at the 2F-HR typing obviously cannot help in allocation decisions but allows optimization of posttransplant patient management by more correct evaluation of the donor specificities of the HLA antibodies. Further efforts should be made to shorten the turnaround time of the 2F-HR typing methods, to make the 2F-HR genotyping results available in the allocation process also for deceased donor transplantation. Finally, due to inaccuracies in the eplet mismatch estimation of the inferred 2F-HR genotypes, mainly for HLA class II, complete 2F-HR HLA genotyping, including DRB₃₄₅, DQA₁ and DPA₁, should be mandatory for clinical use and research on eplet mismatch calculations.

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DISCLOSURE

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









AUTHOR CONTRIBUTIONS

AS, MPE, and MN conceived and designed the study. AS, MPE, VVS, EL, MC, BS, DK, and MN were involved in data collection. AS analyzed the data and prepared the figures and tables. AS and MN drafted the manuscript and all coauthors revised and approved the final version.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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