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ORIGINAL ARTICLE

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# Determination of the clinical relevance of donor epitope-specific HLA-antibodies in kidney transplantation

Tineke Kardol-Hoefnagel[1] | Danial Mohammadi Senejohnny[1] | Elena G. Kamburova[1] | Bram W. Wisse[1] | Leon Reteig[1] | Maartje L. Gruijters[2] | Irma Joosten[3] | Wil A. Allebes[3] | Arnold van der Meer[3] | Luuk B. Hilbrands[4] | Marije C. Baas[4] | Eric Spierings[1,5] | Cornelis E. Hack[1] | Franka E. van Reekum[6] | Arjan D. van Zuilen[6] | Marianne C. Verhaar[6] | Michiel L. Bots[7] | Adriaan C. A. D. Drop[1] | Loes Plaisier[1] | Rowena C. A. Melchers[1] | Marc A. J. Seelen[8] | Jan Stephan Sanders[8] | Bouke G. Hepkema[9] | Annechien J. A. Lambeck[9] | Laura B. Bungener[9] | Caroline Roozendaal[9] | Marcel G. J. Tilanus[10] | Christina E. Voorter[10] | Lotte Wieten[10] | Elly M. van Duijnhoven[11] | Mariëlle A. C. J. Gelens[11] | Maarten H. L. Christiaans[11] | Frans J. van Ittersum[12] | Shaikh A. Nurmohamed[12] | Neubury M. Lardy[13] | Wendy Swelsen[13] | Karlijn A. M. I. van der Pant[2] | Neelke C. van der Weerd[2] | Ineke J. M. ten Berge[2] | Andries Hoitsma[14] | Paul J. M. van der Boog[15] | Johan W. de Fijter[15] | Michiel G. H. Betjes[16] | Dave L. Roelen[17] | Frans H. Claas[17] | Frederike J. Bemelman[2] | Aleksandar Senev[18,19] | Maarten Naesens[18,20] | Sebastiaan Heidt[17] | Henny G. Otten[1,5]

**Correspondence**
Tineke Kardol-Hoefnagel and Henny G. Otten, Center for Translational Immunology, University Medical Center Utrecht, KC02.085.2, P.O. Box 85500, 3508 GA Utrecht, The Netherlands.
Email: t.hoefnagel@umcutrecht.nl and h.g.otten@umcutrecht.nl

In kidney transplantation, survival rates are still partly impaired due to the deleterious effects of donor specific HLA antibodies (DSA). However, not all luminex-defined DSA appear to be clinically relevant. Further analysis of DSA recognizing polymorphic amino acid configurations, called eplets or functional epitopes, might improve the discrimination between clinically relevant vs. irrelevant HLA antibodies. To evaluate which donor epitope-specific HLA

**Abbreviations:** AKME, adjusted Kaplan Meier estimates; CIs, confidence intervals; CPH, cox proportional hazard; DESAs, donor epitope-specific HLA antibodies; DSAs, donor specific HLA antibodies; EURCAU, European Caucasian; HRs, hazard ratios; mAb, monoclonal antibodies; MENAFC, Middle Eastern or North Coast of Africa; NMDP, National Marrow Donor Program; SAB, single antigen bead; SCSEAI, Southeast Asian.

For affiliations refer to page 12

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antibodies (DESAs) are clinically important in kidney graft survival, relevant and irrelevant DESAs were discerned in a Dutch cohort of 4690 patients using Kaplan–Meier analysis and tested in a cox proportional hazard (CPH) model including nonimmunological variables. Pre-transplant DESAs were detected in 439 patients (9.4%). The presence of certain clinically relevant DESAs was significantly associated with increased risk on graft loss in deceased donor transplantations ($p < 0.0001$). The antibodies recognized six epitopes of HLA Class I, 3 of HLA-DR, and 1 of HLA-DQ, and most antibodies were directed to HLA-B (47%). Fifty-three patients (69.7%) had DESA against one donor epitope (range 1–5). Long-term graft survival rate in patients with clinically relevant DESA was 32%, rendering DESA a superior parameter to classical DSA (60%). In the CPH model, the hazard ratio (95% CI) of clinically relevant DESAs was 2.45 (1.84–3.25) in deceased donation, and 2.22 (1.25–3.95) in living donation. In conclusion, the developed model shows the deleterious effect of clinically relevant DESAs on graft outcome which outperformed traditional DSA-based risk analysis on antigen level.

**KEYWORDS**
donor epitope specific antibodies, graft survival, kidney transplantation

## 1 | INTRODUCTION

Kidney transplantation is the best treatment option for end-stage renal failure patients. However, survival rates are still impaired partly due to the presence of pre-transplant donor-specific HLA antibodies (DSAs). The introduction of the single antigen bead assay to define DSAs greatly increased the sensitivity and specificity of antibody detection compared with the classic crossmatch technology. The pre-transplant presence of DSAs as determined by the SAB assay is considered a risk factor for antibody-mediated acute rejection, and graft loss in deceased donation.[1,2]

However, the fact that the majority of patients (64.8%, range 54%–75%)[1–5] with luminex-defined DSAs still have a functioning kidney 10 years post-transplantation, indicates that not all DSAs are harmful. To determine the clinical relevance of DSAs, the MFI on the beads is only a surrogate marker of the antibody level. Determination of antibody levels in patient serum is negatively influenced by the presence of interfering substances and epitope spreading on the beads.[6,7] Furthermore, HLA antibody SAB MFI assessment is not a quantitative assay, and MFI is not equal to antibody titers, as some antibodies with relatively high MFI values may dilute quickly and are therefore not qualified as high titer antibodies (STAR recommendations 2017, 2022).[8,9] The MFI level does not sufficiently predict long-term graft outcome due to well-known limitations of the SAB assay (prozone effect, bead saturation, shared epitope phenomenon, etc.).[10,11] In addition, it has been suggested that especially complement binding DSAs are pathogenic. Although biopsies of rejected grafts showed positive C4d deposition,[12–14] convincing evidence has emerged that antibody-mediated rejection could also occur in absence of positive C4d staining.[15,16] Although studies showed that the specificity of pre-transplant DSAs (Class I and/or Class II) did not clearly impact long-term transplant outcome,[17] there is also research showing that in particular persistent Class II DSAs are associated with inferior graft survival.[18]

To determine which DSAs are clinically important, their precise specificities defined as donor epitope specific antibodies (DESAs) may be important to examine. An eplet or functional HLA epitope—a concept introduced by Duquesnoy[19]—is defined as a cluster of polymorphic amino acid configurations within a 3.0–3.5 Å radius that is needed to induce an antibody response, and that these epitopes may be identical on different HLA molecules.[20,21] Literature showed that the number of HLA epitope mismatches has been associated with poor graft function in different types of solid organ transplantation.[22,23] However, the clinical relevance of DESAs recognizing epitopes shared between different HLA molecules, compared with DSA, has not been defined yet.

Eplet mismatches between patient and donor can result in de novo formation of DSAs[24] and antibody-verified eplet mismatches—according to the HLA Eplet Registry—have shown to be independent predictors of

graft failure.[25] However, large studies regarding the association of pre-existing DESAs with kidney graft loss are lacking. We attempted to fill this knowledge gap by evaluating which DESAs are clinically relevant using a graft survival model in a national-wide consortium of more than 5000 kidney transplant patients. First, we studied the effects on graft survival of several parameters related to the antibody potential to activate complement, including epitope distance to the target cell membrane, number of epitope mismatches, and epitopes verified to bind antibodies.[26–28] As these hypothesis-based methods were not successful in distinguishing clinically relevant from irrelevant DESA, we secondly evaluated whether we could find clinical relevant DESAs based on their relation to graft loss.

## 2 | MATERIALS AND METHODS

### 2.1 | Patients, sera, and clinical data

This study included all 6097 kidney transplants performed between January 1995 and December 2005 in all Dutch transplant centers, and is part of the national PROCARE consortium (PROfiling Consortium on Antibody Repertoire and Effector functions). The complement-dependent cytotoxicity crossmatch assay was negative in all patients. Pre-transplant patient sera could be collected from 4787 transplants. Due to loss to follow-up or kidney failure during surgery or shortly thereafter due to non-immunological reasons, 63 patients were excluded from further analysis.[1] Informed consent for use of their clinical data was obtained from all subjects. The study protocol was approved by the Biobank Research Ethics Committee of the UMC Utrecht (Tc Bio 13-633).

Serum was collected in 2014–2015 from all participating centers, and measured by Luminex. At that time, serum pre-treatment with EDTA was not yet recommended.

### 2.2 | Upscaling HLA typing data

Patients and donors were typed at serological level of resolution for HLA-A, B, and DRB1. In addition, HLA-DQB1 typing was performed in 3582 out of 4690 transplants. As DESA analysis required HLA data at higher resolution these HLA typing data were used for the upscaling process as follows. HLA typings were upscaled using the National Marrow Donor Program (NMDP) haplotype frequency tables,[29] following the algorithm described by Madbouly et al.[30] HLA typings at loci A, B, DRB1, and DQB1 were used as input for the upscaling; upscaled genotypes were derived for loci A, B, C, DRB1, DRB3/4/5, and DQB1.

The NMDP has made haplotype frequency tables available for 21 self-identified race/ethnicity populations in the United States. No information on ethnicity was available for our cohort. Therefore, in line with the Netherlands' ethnical composition from 1995 to 2006 the upscaling procedure considers the most likely ethnicities to be European Caucasian (EURCAU), then Middle Eastern or North Coast of Africa (MENAFC) and Southeast Asian (SCSEAI), and then the other ethnicities, in a stepwise fashion. First, we considered the top 5000 haplotypes for each ethnicity, starting with EURCAU. If no result was found for EURCAU, the MENAFC or SCSEAI genotype with the highest frequency was selected. If still no result was found, the highest frequency genotype among all other populations were selected. If still no matching genotype was found, the typings were manually upscaled using the HaploStats website (https://haplostats.org). Due to missing HLA typing data in 34 transplants (22 donors, 12 recipients), those transplants were excluded, resulting in a total of 4690 transplants included in the analysis, with a high-resolution genotype for each donor–recipient pair. Geneugelijk et al[31] developed a computational method using a representative recipient population consisting of all HLA genotyping data performed at the University Medical Center Utrecht between 2009 and 2016, and of a virtual Caucasian donor population consisting of 10 million individuals based on HLA haplotype frequency tables from NMDP 2007 to 2011, as we did. They compared calculated eplet values based on serological split HLA typing data (observed values) to two-field resolution HLA genotyping data (reference values). It is expected that the effect of eplet numbers on alloreactivity is more logarithmic than linear, and therefore the eplet values were converted into the natural logarithm thereof (e.g., ln (eplet)). 87.7% of observed ln(eplet) values deviated maximal 0.1, including about 1/3 (28.3%) to be identical to reference values.

### 2.3 | Determination of epitope distance to the cell membrane

The mean distance of epitopes to the cell membrane was found as follows. First, the (x, y, z) coordination of the tail of HLA was determined. Then the Euclidean distance (norm-2) between the average (x, y, z) location of epitopes and the coordination of the tail of HLA was found. The mean distance was taken because each epitope is comprised of more amino acids and atoms and the location of all of them needed to be average to find the center of the given epitope.

## 2.4 | Detection and definition of DESAs

To define antibodies against a donor specific epitope, pre-transplant heat-inactivated patient sera long-term stored at −20°C or colder were tested for the presence of HLA Class and/or Class II antibodies using LifeCodes SAB assay Class I and/or II kits (Immucor Transplant Diagnostics, Stamford, CT). Bead positivity was defined according to the manufacturer's instructions. In short, 3 values were to be calculated: (1) BCM value (raw MFI value minus a lot-specific background MFI per bead); (2) BCR ratio (BCM divided by the lowest MIF of all beads with antigens of the same locus); (3) AD-BCR ratio (BCR divided by the relative amount of antigen coated on the bead). The bead is deemed positive when 2 of the 3 values are above a lot-specific threshold, respectively, 1500, 3, and 4. Next, all eplets were assessed in the HLA Eplet Registry (epregistry.com.br, accessed December 2, 2022) (in total 492 eplets). The HLA typing's for donor and recipient were converted to epitopes, and the epitopes belonging only to the donor that were absent on the recipients were used (mismatched epitopes). Also, the results of the SAB assay were converted into epitopes. DESAs were defined as mismatched donor epitopes present only on the positive beads containing these epitopes for HLA Class I (HLA-A, -B, -C) and/or Class II (HLA-DRB1, -DRB345, -DQA1, -DQB1) (Figure S1). If a mismatched epitope occurs on a negative bead, it is not included in the definition of a DESA. To visualize the recognition sites of DESA on HLA molecules, a plotly dash application in dash bio package was used (Molecule3dViewer) with python 3.8.

## 2.5 | Investigation and validation cohort

To exclude that DESAs related with graft loss were defined by chance, it has been investigated whether these DESAs can be found in independent cohorts. For this purpose, the deceased donor dataset was randomly split 100 times into a 70% investigation and a 30% validation cohort. This approach has been published previously, and employing a random sampling with at least 100 repeats and a reasonable balance between investigation and validation cohort (50%–70% for investigation), was likely to get a good reliable model, as described by Xu and Goodacre.[32,33] DESAs that were frequent enough in each cohort (at least twice) were considered. For each specific DESA in the investigation cohort, adjusted Kaplan–Meier estimates (AKME) for transplants with one specific DESA against all other DESA were plotted. Specific DESA that showed on average 10% difference in 10-years graft survival between these AKMEs were selected. This procedure was repeated for the specific DESA in the validation cohort. A given DESA was considered clinically relevant whenever it satisfied the chosen metric, that is, average 10% difference over 10-year between AKMEs, in both the investigation and validation cohort for at least 30 times out of 100 try. Subsequently, the results found in deceased donors were applied to the living donors.

## 2.6 | Statistical analysis

Patient, donor, and transplant characteristics in presence and absence of DESA was evaluated for both continuous and categorical variables via the Mann–Whitney $U$ test and $\chi 2$ test, respectively. Kaplan–Meier estimator was employed for death-censored graft survival analysis, where the curves are adjusted for a number of covariates based on inverse probability weighting.[34] The covariates for which we adjusted are recipient age (quadratic) and donor age (quadratic), donor type (living or deceased; for the total cohort only), CIT (for donation after brain death and donation after cardiac death), time on dialysis in years (quadratic), and induction therapy with IL-2 receptor blocker. Other covariates were not used for various reasons as previously described by Kamburova et al[1] A $p$-value <0.05 was considered statistically significant.

Hazard ratios (HRs) and confidence intervals (CIs) were derived by using multivariable Cox regression. Validity of Cox model assumptions were verified by evaluating Schoenfeld residual plots. Statistical analyses were performed with python 3.8, R version (3.2.2) and SAS (version 9.4; SAS Institute, Cary, NC) software.

# 3 | RESULTS

## 3.1 | Baseline characteristics

Patient, donor, and transplant characteristics stratified according to the presence of pre-transplant DESAs are summarized in Table 1. 439 patients (9.4%) of 4690 patients had pre-transplant DESAs. DESAs were detected only in renal transplant recipients positive for donor specific HLA. In the total cohort, 3398 patients (72.5%) were completely HLA-ab negative. The DESA group contained a higher proportion of female recipients (60.8% [$n = 267/439$] vs. 32.8% [1115/3398] in the HLA-ab negative group), and PRA values were related to the presence of DESAs. Recipients with DESAs received more often hemodialysis (58.3% [256/439] vs. 49.2% [1672/3398] in patients without HLA-ab). Additionally, there were significantly more retransplants in the DESA group (45.6% [200/439] vs. 4.5% [153/3398]). Minimal follow-up time was 10 years after transplant.

**TABLE 1** Patient, donor, and transplant characteristics.

| Characteristics | No HLA Ab ($n = 3398$) | No DESAs ($n = 853$) | DESAs ($n = 439$) | $p$-Value |
|---|---|---|---|---|
| Patient | | | | |
| Age at transplant, *y*, mean ± SD | 45.6 ± 14.6 | 45.1 ± 13.7 | 44.6 ± 13.7 | 0.270[a] |
| Female sex, *n* (%) | 1115 (32.8) | 497 (58.3) | 267 (60.8) | <0.001[b] |
| PRA at time of transplant, %, mean ± SD | 0.9 ± 4.3 | 16.2 ± 26.3 | 25.6 ± 31.7 | <0.001[a] |
| Highest PRA, % mean ± SD | 4.4 ± 10.7 | 36.1 ± 34.9 | 43.8 ± 36.8 | <0.001[a] |
| Dialysis | | | | |
| No, *n* (%) | 414 (12.2) | 68 (8.0) | 44 (10.0) | <0.001[b] |
| Yes-hemodialysis, *n* | 1672 (49.2) | 505 (59.2) | 256 (58.3) | |
| Yes-peritoneal dialysis, *n* | 1295 (38.1) | 276 (32.4) | 132 (30.1) | |
| Unknown, *n* (%) | 17 (0.5) | 4 (0.5) | 7 (1.6) | |
| Time on dialysis, *y*, mean ± SD | 2.5 ± 2.2 | 3.4 ± 3.0 | 3.3 ± 3.0 | <0.001[a] |
| Donor | | | | |
| Donor age, *y*, mean ± SD | 44.4 ± 15.1 | 43.5 ± 14.6 | 45.1 ± 15.3 | 0.163[a] |
| Donor female sex, *n* (%) | 1761 (51.8) | 412 (48.3) | 193 (44.0) | 0.003[b] |
| Type of donor | | | | <0.001[b] |
| Living, *n* (%) | 1147 (33.8) | 184 (21.6) | 124 (28.2) | |
| Deceased-DBD, *n* (%) | 1628 (47.9) | 551 (64.6) | 248 (56.5) | |
| Deceased-DCD, *n* (%) | 623 (18.3) | 118 (13.8) | 67 (15.3) | |
| Cold ischemia time | | | | |
| Deceased donors, *h*, mean ± SD | 21.4 ± 7.3 | 23.1 ± 6.9 | 22.6 ± 7.0 | <0.001[a] |
| Living donors, *h*, mean ± SD | 2.5 ± 1.6 | 2.5 ± 1.1 | 2.5 ± 1.0 | 0.989[a] |
| Transplant | | | | |
| Retransplant, *n* (%) | 153 (4.5) | 367 (43.0) | 200 (45.6) | <0.001[b] |
| HLA-A/B/DR/DQ broad mismatches, mean ± SD | 2.8 ± 1.8 | 2.6 ± 1.7 | 2.7 ± 1.7 | 0.027[a] |
| Induction therapy | | | | |
| IL-2 receptor blocker, *n* (%) | 768 (22.6) | 161 (18.9) | 86 (19.6) | 0.034[b] |
| T cell-depleting antibody[c], *n* (%) | 100 (2.9) | 54 (6.3) | 30 (6.8) | <0.001[b] |
| Initial immunosuppression, *n* (%) | | | | |
| Steroids | 3331 (98.0) | 831 (97.4) | 423 (96.4) | 0.063[b] |
| MMF/azathioprine | 2583 (76.0) | 644 (75.5) | 350 (79.7) | 0.192b |
| Cyclosporine/tacrolimus | 3179 (93.6) | 808 (94.7) | 415 (94.5) | 0.368[b] |
| Sirolimus | 210 (6.2) | 51 (6.0) | 23 (5.2) | 0.735[b] |
| Other | 478 (14.1) | 87 (10.2) | 35 (8.0) | <0.001[b] |
| Unknown | 11 (0.3) | 1 (0.1) | 4 (0.9) | 0.064[c] |

Abbreviations: DBD, donation after brain death; DCD, donation after cardiac death; DESA, donor epitope specific antibody; IL, interleukin; MMF, mycophenolate mofetil.

[a]One-way ANOVA for continuous variables.

[b]Chi-square test for categorical variables.

[c]T cell-depleting antibody therapy: ALG, ATG, OKT3 monoclonal antibodies.

Pre-transplant DESAs include 161 eplets of HLA Class I, 95 eplets of HLA-DR, and 57 eplets of HLA-DQ (Table S1). The median number of donor eplets to which patients have antibodies is 6 (range 1–34). Of 439 transplants most eplets are directed to 1 locus (HLA-A 83, -B 80, -C 17, -DR 86, and 63 HLA-DQ). Eighty-six transplants (20%) have DESAs directed to 2 loci, and 24 (5%) directed to 3 loci (Table 2).

**TABLE 2** Characteristics of donor eplets per HLA Class.

| | HLA-A, -B, -C | HLA-DR | HLA-DQ | Total |
|---|---|---|---|---|
| Epitopes listed in HLA epitope Registry | 224 | 123 | 83 | 430 |
| Donor epitopes | 162 | 94 | 57 | 313 |
| No. of transplants with DESA | | | | 439 |
| **Donor epitopes** | **No. (%) of transplants** | | | |
| Number per patient | | | | |
| 1 | 82 (18.7) | | | |
| 2 | 63 (14.4) | | | |
| 3 | 51 (11.6) | | | |
| 4 | 44 (10) | | | |
| 5 | 40 (9.1) | | | |
| >5 | 159 (36.2) | | | |
| Directed to 1 locus | | | | |
| A | 83 (18.9) | | | |
| B | 80 (18.2) | | | |
| C | 17 (3.9) | | | |
| DR | 86 (19.6) | | | |
| DQ | 63 (14.4) | | | |
| Directed to 2 loci | | | | |
| A, B | 20 (4.6) | | | |
| A, C | 6 (1.4) | | | |
| A, DQ | 6 (1.4) | | | |
| A, DR | 12 (2.7) | | | |
| B, C | 8 (1.8) | | | |
| B, DQ | 1 (0.23) | | | |
| B, DR | 8 (1.8) | | | |
| C, DQ | 3 (0.68) | | | |
| C, DR | 1 (0.23) | | | |
| DR, DQ | 21 (4.8) | | | |
| Directed to 3 loci | | | | |
| A, B, C | 2 (0.46) | | | |
| A, B, DR | 5 (1.1) | | | |
| A, B, DQ | 1 (0.23) | | | |
| A, C, DR | 1 (0.23) | | | |
| A, C, DQ | 1 (0.23) | | | |
| A, DR, DQ | 5 (1.1) | | | |
| B, C, DR | 1 (0.23) | | | |
| B, C, DQ | 2 (0.46) | | | |
| B, DR, DQ | 5 (1.1) | | | |
| C, DR, DQ | 1 (0.23) | | | |

Abbreviation: DESA, donor epitope specific antibody.

## 3.2 | Hypothesis-based parameters associated with clinical impact of DESA

Since in the same cohort previous data showed a significant association between pre-transplant DSAs and graft survival only in deceased donors,[1] we investigated the possible clinical relevance of DESA in a cohort of 3235 kidney transplant recipients receiving a deceased donor graft. Clinically relevant DESA is defined as those DESA that resulted in significant impaired graft survival. We first evaluated epitope distance to the cell membrane, number of DESAs present in transplants, and epitopes verified to be able to bind antibodies, in relation to 1-year and 10-year graft survival. No significant differences were found between distance of those epitopes from non-failed transplants ($n = 286$) and transplants that failed in the first-year post-transplantation ($n = 205$) (Figure S2A,B). Although an inferior graft survival in patients with DESAs compared with patients without DESAs was observed, the number of DESA did not result in significant differences in graft survival (Figure S2C). Finally, we performed an analysis based on the experimentally antibody-verified epitopes reported by Bezstarosti et al.[35] We hypothesized that DESAs recognizing antibody-verified epitopes are more clinically relevant than those recognizing non-verified epitopes. Analysis of transplants with one or more of these DESAs recognizing mAb showed no difference between experimentally verified DESAs and not antibody-verified DESAs (Figure S2D).

## 3.3 | Determination and characteristics of clinically relevant DESA

Since hypothesis-based parameters did not improve the distinction between clinically relevant from irrelevant DESA, we applied a different approach. We analyzed the total cohort, in which we found 10 unique DESAs (3.2% of donor epitopes found in the current study) being related to more than 10% graft loss compared with transplants without DESAs (DESA related to graft loss) (Figure 1, red dots). These included 6 eplets of HLA Class I (71TD, 76ED, 76ET, 80TLR, 144QL, 158T), 3 eplets of HLA-DR (67F, 70DA, 70DRA), and 1 eplet of HLA-DQ (45EV). Seventy-six patients had one or more DESA related to graft loss, and the majority of patients (53 [69.7%]) recognized one clinically relevant donor eplet (range 1–5). Most of those DESAs were directed to one locus: 36 (47%) transplants to HLA-B (1 to -A, 0 to -C), 10 (13.2%) transplants to HLA-DR, and 28 (36.8%) to HLA-DQ (Table 3). No differences in MFI were observed between clinically relevant and irrelevant

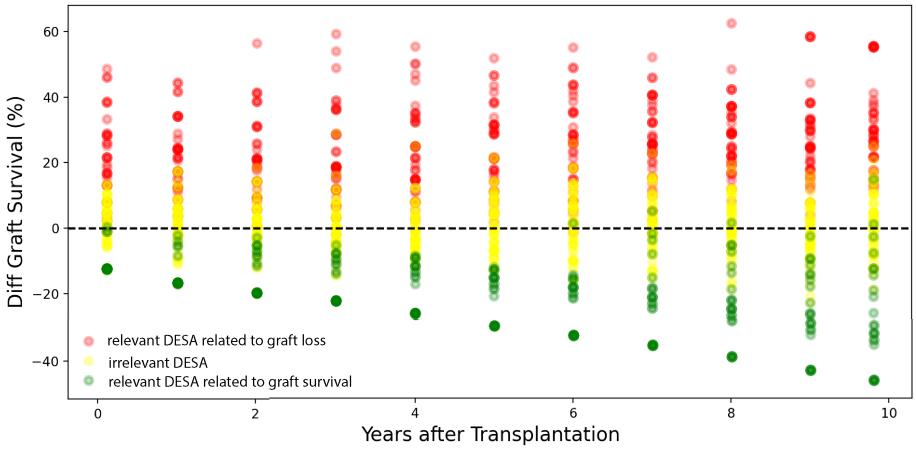


**FIGURE 1** Overview of clinically relevant and irrelevant donor epitope specific antibodies (DESA) in deceased transplants displayed by their individual effect on graft survival. Differences in graft survival of a specific DESA against other DESAs are displayed. Each dot depicted an individual epitope recognized by antibodies. We considered a difference in 10-year death-censored graft survival of 10% or less to be clinically irrelevant (yellow dots), plotted in an adjusted Kaplan–Meier estimate. DESA resulting in an increased risk on graft loss (>0.1) are displayed in red (DESA related to graft loss), while DESA resulting in a decreased risk (<−0.1) are displayed in green (DESA related to graft survival).

DESAs in pre-transplant sera. We also found some clinically relevant DESAs related to improved graft survival (Figure 1, green dots).

We also looked at the effect of using a less strict cut-off value, for example 7%, and of including all clinically relevant DESAs whether or not they were present in one or more transplants. More clinically relevant donor eplets were found, and the effect on graft survival remained quite similar (10-year death-censored graft survival of around 40% for patients with clinically relevant DESAs for deceased donor transplants, and 70%–75% for living donor transplants). We have observed quite a few clinically relevant donor eplets that are present only in one or a few transplants, indicating that very large numbers are needed to find those eplets in a larger number of transplants.

### 3.4 | Association of clinically relevant DESA related to graft loss

The combined effect was examined of clinically relevant individual DESAs found in the complete cohort. For deceased donor transplants ($n = 3235$), the AKME showed a 10-year death-censored graft survival of 32%, 59%, and 76% for patients with clinically relevant DESAs individually related to graft loss, with other DESAs, and without DESAs, respectively (Figure 2A; $p < 0.0001$), demonstrating a much higher adverse effect of those DESAs on graft loss compared with the effect of DSAs in the same cohort.[1] For the living donor transplants ($n = 1455$), the relation between clinically relevant DESAs individually related to graft loss and 10-year death-censored graft survival was also significant ($p = 0.0002$), which was 58% for patients with DESAs related to graft loss and 84% for patients without DESAs (Figure 2B). For the deceased donor transplants, we observed a significant effect of the clinically relevant DESAs on graft loss in both the short- and long-term (Figure 2C,E; $p < 0.002$), in contrast to the living donor transplants that showed only a significant effect on the short-term (Figure 2D,F). These findings were confirmed in a multivariable analysis (Table 4), adjusted for the same covariables. The presence of clinically relevant DESAs was significantly associated with a higher risk of graft loss after deceased donations (HR 2.45, 95% CI 1.84–3.25), and in living donor transplants (HR 2.22, 95% CI 1.25–3.95). Investigation where these epitopes are located on HLA molecules indicated that most of the epitopes recognized by clinically relevant DESA are located on the top of an HLA molecule (Figure 3).

## 4 | DISCUSSION

In this study, we evaluated the clinical relevance of DESAs in a large cohort of kidney transplant recipients. Using a hypothesis-free approach by calculating the difference in graft survival between a certain DESA and others, resulted in a model in which clinically relevant DESAs could be distinguished from irrelevant DESAs. 10-year death-censored graft survival is only 32% for patients with clinically relevant DESAs in deceased donor transplants, and 58% in living donor transplants. The results were confirmed in a multivariable Cox regression analysis.

**TABLE 3** Characteristics of clinically relevant donor epitopes per HLA Class.

| | HLA-A, -B, -C | HLA-DR | HLA-DQ | Total |
|---|---|---|---|---|
| Epitopes listed in HLA epitope registry | 224 | 123 | 83 | 430 |
| Clinically relevant donor epitopes | 6 | 3 | 1 | 10 |
| No. of transplants with clinically relevant DESA | | | | 76 |
| **Clinically relevant donor epitopes** | | | | **No. (%) of transplants** |
| Number per patient | | | | |
| 1 | | | | 53 (69.7) |
| 2 | | | | 9 (11.8) |
| 3 | | | | 9 (11.8) |
| 4 | | | | 4 (5.3) |
| 5 | | | | 1 (0.01) |
| Directed to 1 locus | | | | |
| A | | | | 1 (0.01) |
| B | | | | 36 (47.4) |
| C | | | | 0 |
| DR | | | | 10 (13.2) |
| DQ | | | | 28 (36.8) |
| Directed to 2 loci | | | | |
| B, DQ | | | | 1 (0.01) |

Abbreviation: DESA, donor epitope specific antibody.

To define eplet mismatches between donors and recipients, several algorithms are available (e.g., HLA Matchmaker, OLI Fusion Matchmaker), but there are differences in eplet definitions between those algorithms.[35,36] Moreover, eplet definitions have been subject to change, especially for HLA-DQ,[21] indicating that there is no definitive and accepted list of eplets yet. Finally, high resolution typing data are required for optimal DESA analysis. These data were not available in our cohort, and therefore split-serological typing were up scaled to high-resolution typing. Imputation could result in less accurate high-resolution typing. Others that used the same upscaling approach showed different results in terms of accuracy. Madbouly et al[30] suggested a 65%–70% accuracy for Caucasians, and Geffard et al[37] showed an accuracy of 58%. Engen et al[38] tested HaploStats accuracy in a multi-ethnic population, and translating imputation output of high-resolution data when entering low-resolution HLA typing data into eplets resulted in incorrect eplet identification in about 23% of the Caucasian population,[38] and errors were more common in the non-Caucasian population. Ferradji et al[39] described that the NMDP HaploStats imputation tool yielded a high prediction accuracy for HLA Class I (allele-level recall values >95%), and a moderate accuracy for HLA Class II (allele-level recall values >80%) in Caucasians. Senev et al[40] described that considering the eplet repertoire the agreement between the imputated and real high-resolution typing data was 75% for HLA Class I and only 35% for Class II, indicating that for the interpretation of the data the lower imputation accuracy should be taken into account especially for HLA Class II molecules, and in a non-Caucasian population.

To the best of our knowledge, this is the first time that a model showed significantly inferior graft survival based on clinical relevance of DESA. Although multicenter studies found poor graft survival in recipients with pre-transplant DSAs transplanted with either living or deceased donors, the 5-year and 10-year graft survival is still between 60% and 70%,[1,4] while in our study the 10-year graft-survival is 32% in DESA positive patients. In the current cohort, the percentage of patients with pre-transplant DSAs is comparable to pre-transplant DESAs (12% and 9.4%, respectively). The presence of pre-transplant clinically relevant DESA has detrimental effect on early graft survival and also on late graft survival in deceased donation. We did not try to identify clinical relevant DESAs simply by identifying patients with early graft loss, because the number of those patients is too low to discern clinically relevant DESAs ($n = 319$ graft loss <6 months). Whether the described approach to classify clinically relevant and nonrelevant DESA is also applicable to classify DSAs into clinically relevant and nonrelevant antibodies, could be investigated in future research. We would like to emphasize that it is very important to note that the distinction between clinically (ir)relevant DESAs is only allowed if pre-transplant luminex DSAs are not a contraindication for transplantation. This is certainly not the case in every center worldwide, as some centers allow only very low-grade pretransplant clinically insignificant DSA to proceed with transplantation. In the current study, transplantations were not biased by pre-transplant Luminex results, with neat MFI levels of pre-transplant DSA varying between 500 up and >10.000.[1] Furthermore, the cohort consisted of relatively low immunological risk patients as transplants only occurred after a negative crossmatch, highlighting specifically the effect of lower level pre-transplant DSAs.

It appeared that some transplants were DSAs positive but were DESAs negative (33% of DESA positive transplants). The reason for this discrepancy is that for DSA determination low-resolution typing has been used, and upscaled high-resolution HLA typing data for DESA

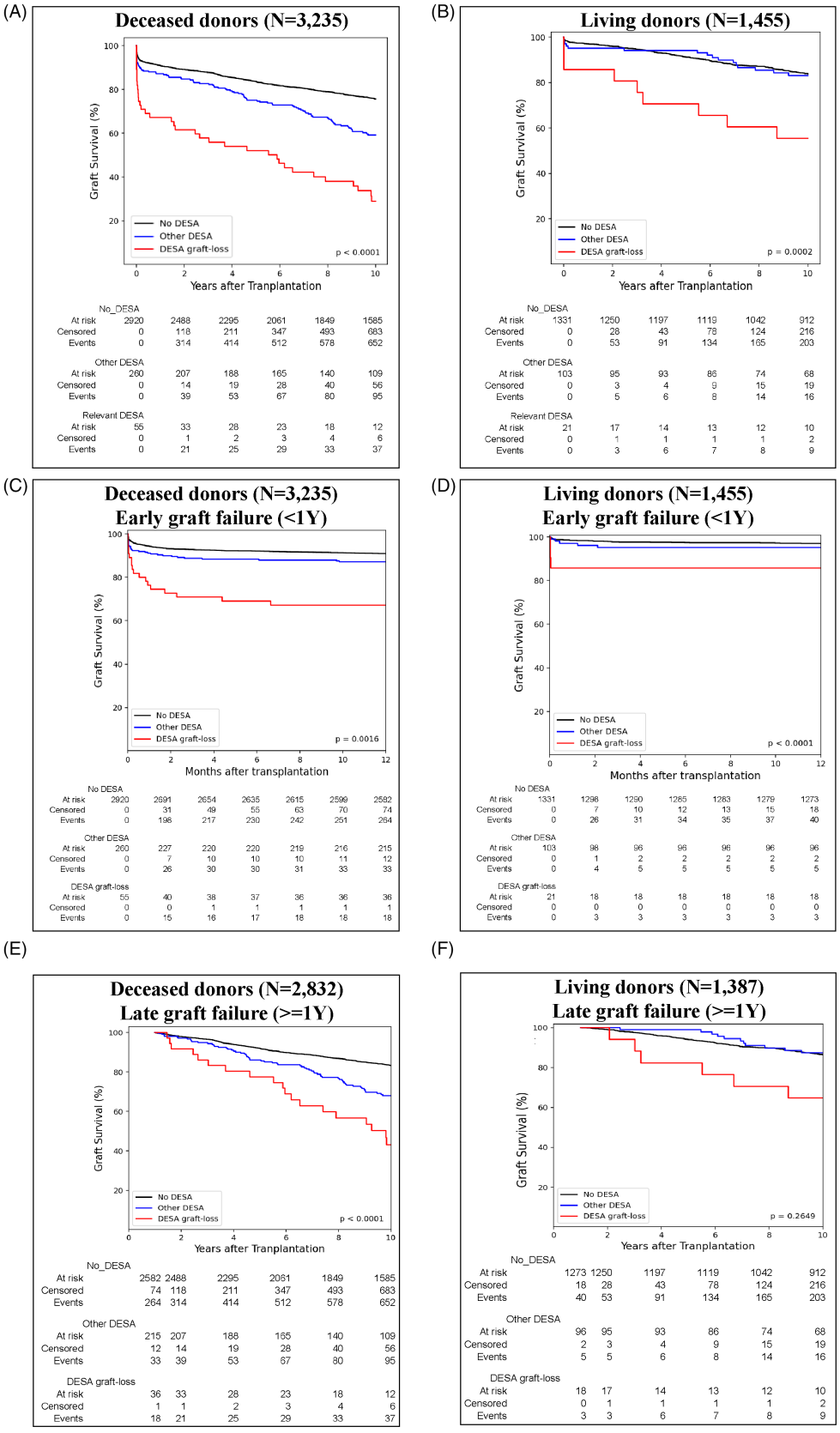


**FIGURE 2** Long-term graft survival of kidney transplants according to clinically relevant donor epitope specific antibodies (DESA) found at least 30 times in both investigation and validation cohort. Adjusted Kaplan Meier estimates (AKME) for death-censored graft survival according to the presence of clinically relevant DESA related to graft loss for the deceased-donor transplants only ($N = 3235$) (A) and for living-donor transplants only ($N = 1455$) (B). Analysis of the effect of clinically relevant DESA related to graft loss on 1-year graft survival for deceased-donor transplant only (C, $N = 3235$) and for living-donor transplants only (D, $N = 1455$). (E) Analysis of long-term effect of clinically relevant DESA related to graft loss starting at 1 year after transplants for deceased-donor transplants only ($N = 2832$) and for living-donor transplants only (F, $N = 1387$). All AKME were adjusted for the following covariates: recipient age (quadratic) and donor age (quadratic), donor type (living or deceased; for the total cohort only), cold ischemia time (for donation after brain death and donation after cardiac death), time on dialysis in years (quadratic), and induction therapy with interleukin-2 receptor blocker.

determination. For example, a DSA has been determined against B44 (donor HLA, serological level) with the corresponding Luminex bead *B*44:03* being positive. After upscaling, it turned out that the donor HLA was *B*44:02* on 2-field resolution level, and therefore no DESA could be determined. 10-year graft survival between transplants

**TABLE 4** Multivariable analyses of DESAs associated with graft loss using Cox proportional hazards model.

| | No. (%) of transplants with DESAs associated with graft loss | Hazard ratio | 95% CI |
|---|---|---|---|
| Total cohort ($N = 4690$) | 76 (1.6) | 2.31 | 1.79–2.98 |
| Living donors ($N = 1455$) | | | |
| All | 21 (1.4) | 2.22 | 1.25–3.95 |
| Early failures (<1 year) | 3 (0.2) | 17.97 | 4.35–74.34 |
| Late failures (≥1 year) | 18 (1.2) | 1.96 | 1.01–3.80 |
| Deceased donors ($N = 3235$) | | | |
| All | 55 (1.7) | 2.45 | 1.84–3.25 |
| Early failures (<1 year) | 36 (1.1) | 2.13 | 1.48–3.07 |
| Late failures (≥1 year) | 19 (0.6) | 1.59 | 1.00–2.54 |

Abbreviations: CI, confidence interval; DESA, donor eplet specific antibody.

*Note*: In this multivariable analysis, we adjusted for differences in the following covariates: recipient age (quadratic), donor age (quadratic), donor type (living or deceased), cold ischemia time in hours for donation after brain death (DBD) and donation after cardiac death (DCD), time on dialysis in years (quadratic), and induction therapy with interleukin-2 receptor-blocking antibody. Other covariates were not used for various reasons as previously described by Kamburova et al (*AJT* 2018).

with DSAs without DESAs is comparable to transplants with both DSAs and DESAs (Figure S3A).

Preliminary data indicate that patients having two or more pre-transplant DESAs, defined by taking all eplets listed in the HLA Eplet Registry into account, showed inferior graft survival compared with patients with zero to two DESAs.[41] However, 10-year graft survival rate is ~50% in patients with ≥2 DESA. Since 54%–75% of the kidney transplant recipient population had still a functioning graft 10 years after transplantation despite the presence of pre-transplant DSAs or DESAs, not all DESAs appear to be equally harmful. This provides room for local policy making as there is no consensus how to take these data into consideration upon an organ offer.

The metric cut-off of 10% used to distinguish clinically relevant from irrelevant DESAs is quite strict, resulting in a small number of relevant donor eplets. The value of 10% is based on, firstly, the average difference in graft survival between patients with and without DSAs in a large cohort.[1] Second, it is based on the data in Figure 1, showing that 10% graft survival difference lies in the middle of the range from 0 to the maximum effect found. The results have been validated both internally (cross-validation) and externally (living donors). The association of these clinically relevant DESA with graft loss was found to be stronger in deceased compared with living transplants, especially early after transplantation, resembling the previously described relation between DSA and graft loss.[1]

The reason why antibodies against certain epitopes are clinically more relevant than others is unknown. Data obtained in therapeutic antibody enhancement studies showed that binding of the Fc region of the antibody to the complement protein C1q is important for activation. In addition, the ability to activate the complement system is dependent on antigen density, size, and the formation of ordered antibody hexamers that efficiently bind C1q.[26–28,42] Finally, it could be that multiple low-level MFI DESAs—that have not been found as complement-fixing—cluster on cell surfaces and therefore are able to activate complement in vivo.[43] The cited literature describes data regarding known epitopes on CD20 and CD137, but we hypothesized that the same parameters may also affect HLA antibody effector functions as many HLA epitopes has been discovered. However, we have to keep in mind that HLA antigens are highly polymorphic.

In the present study, neither epitope distance to the cell membrane, number of DESAs nor antibody-verified epitopes were useful parameters to distinguish clinically relevant from irrelevant DESAs with respect to graft survival. In addition, we observed a dispersed distribution of the clinically relevant DESAs associated with graft loss on HLA molecules, although some eplets contained overlapping amino acids. This suggests that certain positions on HLA molecules are of more importance for the clinical relevance of DESAs than other positions. We would like to emphasize that which HLA antigen specificities will be recognized by clinically (ir)relevant DESAs is not important, as epitopes can be located on multiple HLA antigens. Furthermore, these data suggested that probably complement-independent mechanisms such as binding of innate immune cells to antibodies or direct activation of antibodies also determine the clinical

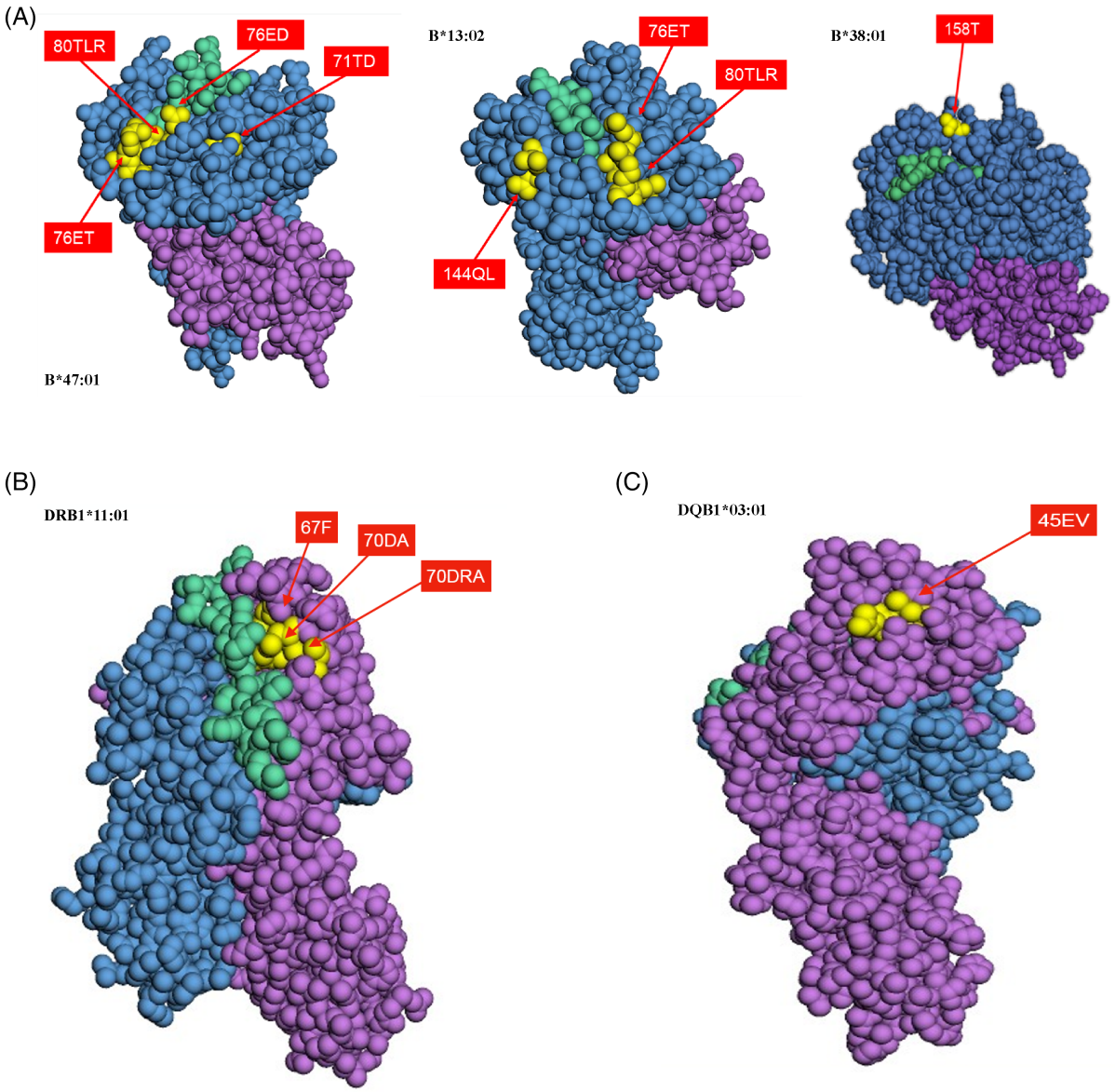


**FIGURE 3** Virtualization of an HLA molecule showing epitopes recognized by clinically relevant donor epitope specific antibodies (DESA). Side view of major histocompatibility complex protein *HLA-B*47:01*, *B*13:02*, and *B*38:01* (A). *HLA-B*47:01* has four eplets: 71TD, 76ET, 76ED, 80TLR; *HLA-B*13:02* has three eplets: 76ET, 80TLR, 144QL; *HLA-B*38:01* has one eplet: 158T. Blue: alpha chain; purple: beta-2 microglobulin chain; green: a presented peptide; yellow: clinically relevant DESA. (B) Side view of major histocompatibility complex protein *HLA-DRB1*11:01* having three eplets: 67F, 70DA, 70DRA, and of protein *HLA-DQB1*03:01* (C) having one eplet: 45EV. Blue: alpha chain; purple: beta chain; green: a presented peptide; yellow: clinically relevant DESA.

relevance of DESAs. Further research is needed to study the effector functions of the clinically relevant DESAs. We have not considered data regarding HLA-DP antibodies. It has been shown that preformed HLA-DP antibodies did not significantly worsen graft survival,[44] therefore we do not think that eliminating patients with preformed anti-DP antibodies would result in different data. We have not investigated the contribution of de novo antibodies to late graft survival nor the immunogenicity of epitopes recognized by clinically relevant DESAs, which is a limitation of the study. Limited information was available on (the registration of) rejections and the type of rejection as well as the reason for graft loss, and as the retrospective cohort consisted of kidney transplants between 1995 and 2005, biopsies were not always performed at that time, and, therefore, rejections might have been missed.

Overall, clinically relevant DESAs did not have higher MFI values than clinically irrelevant DESAs had. As MFI can only be provided against 1 HLA molecule, it is difficult to average that epitope specificity, because DESAs recognize epitopes present on several HLA molecules,

and serum contained several HLA antibodies. In addition to clinically relevant DESAs related to graft loss, other clinically relevant DESAs appeared to be beneficial (Figure 1, green dots). One hypothesis why certain pre-existing antibodies might be beneficial on the long term is via a process called accommodation, known to occur in ABO-incompatible transplants. A possible mechanism could be that inhibition of inflammation through some complement split products might contribute to superior graft survival in patients with those DESAs.[45] It has been shown in animal studies that membrane regulatory complement proteins can be upregulated by antibodies.[46,47]

The major advantage of the multicenter ($n = 8$) patient cohort used is the long follow-up time of minimal 10 years post-transplant, and its size. We observed no differences in graft survival between patients transplanted in 1995–2000 versus in 2000–2005 (Figure S3B). In addition, the proportion of patients with clinically relevant DESAs in both periods were equal (1.9%, and 1.4%, respectively). Furthermore, the analysis for HLA-antibodies defined by Luminex was performed in a single center excluding variances among centers which could negatively influence the data.[48] A donor antibody against a specific epitope was considered clinically relevant if the difference in graft survival between transplants with and without that specific DESA was at least 10%. In the Kaplan–Meier survival analysis, patients with at least one clinically relevant DESA may also have irrelevant DESA, which could average overall graft survival. Due to the low number of transplants with clinically relevant DESAs, we were not able to evaluate the contribution of only HLA Class I or Class II DESAs to graft survival, neither to analyze whether increasing number of clinically relevant DESAs is related to inferior graft survival. In addition, as the cohort mainly consisted of Caucasians, the results definitely need to be replicated in other cohorts from different countries including non-European populations.

In conclusion, we developed a model that associated pre-existing DESAs appearing to be clinically relevant in relation to graft survival. Certain specific DESA are more clinically relevant than others in both living- and deceased-donor transplants resulting in a 10-year graft survival of 32% in patients with those DESAs. These results can be used for pre-transplant risk stratification for graft loss and improves the current state-of-the-art where DSA instead of DESA are used.

## AUTHOR CONTRIBUTIONS

Danial Mohammadi Senejohnny and Henny G. Otten designed the study. Danial Mohammadi Senejohnny performed the data analysis. Michiel L. Bots was involved in interpretation of the data. Aleksandar Senev and Maarten Naesens provided data from another cohort. Tineke Kardol-Hoefnagel wrote the manuscript. All other authors (Elena G. Kamburova, Bram W. Wisse, Maartje L. Gruijters, Irma Joosten, Wil A. Allebes, Arnold van der Meer, Luuk B. Hilbrands, Marije C. Baas, Eric Spierings, Cornelis E. Hack, Franka E. van Reekum, Arjan D. van Zuilen, Marianne C. Verhaar, Adriaan C. A. D. Drop, Loes Plaisier, Rowena C. A. Melchers, Marc A. J. Seelen, Jan Stephan Sanders, Bouke G. Hepkema, Annechien J. A. Lambeck, Laura B. Bungener, Caroline Roozendaal, Marcel G. J. Tilanus, Christina E. Voorter, Lotte Wieten, Elly M. van Duijnhoven, Mariëlle A. C. J. Gelens, Maarten H. L. Christiaans, Frans J. van Ittersum, Shaikh A. Nurmohamed, Neubury M. Lardy, Wendy Swelsen, Karlijn A. M. I. van der Pant, Neelke C. van der Weerd, Ineke J. M. ten Berge, Andries Hoitsma, Paul J. M. van der Boog, Johan W. de Fijter, Michiel G. H. Betjes, Dave L. Roelen, Frans H. Claas, Frederike J. Bemelman, Sebastiaan Heidt) were involved in recruiting patients and collecting patient material. All authors gave final approval of the version to be submitted.

## AFFILIATIONS

[1]Center for Translational Immunology, University Medical Center Utrecht, Utrecht, The Netherlands
[2]Renal Transplant Unit, Department of Internal Medicine, Amsterdam University Medical Center, Amsterdam, The Netherlands
[3]Laboratory Medicine, Laboratory of Medical Immunology, Radboud University Medical Center, Nijmegen, The Netherlands
[4]Department of Nephrology, Radboud University Medical Center, Radboud Institute for Health Sciences, Nijmegen, The Netherlands
[5]Central Diagnostic Laboratory (CDL), University Medical Center Utrecht, Utrecht, The Netherlands
[6]Department of Nephrology and Hypertension, University Medical Center Utrecht, Utrecht, The Netherlands
[7]Julius Center for Health Sciences and Primary Care, University Medical Center Utrecht, Utrecht University, Utrecht, The Netherlands
[8]Department of Nephrology, University Medical Center Groningen, Groningen, The Netherlands
[9]Department of Laboratory Medicine, University Medical Center Groningen, Groningen, The Netherlands
[10]Department of Transplantation Immunology, Tissue Typing Laboratory, Maastricht University Medical Center, Maastricht, The Netherlands
[11]Department of Internal Medicine, Division of Nephrology, Maastricht University Medical Center, Maastricht, The Netherlands
[12]Department of Nephrology, Amsterdam University Medical Center, Amsterdam, The Netherlands
[13]Department of Immunogenetics, Sanquin Diagnostic Services, Amsterdam, The Netherlands

[14]Dutch Organ Transplant Registry (NOTR), Dutch Transplant Foundation (NTS), Leiden, The Netherlands
[15]Department of Nephrology, Leiden University Medical Center, Leiden, The Netherlands
[16]Department of Nephrology, Erasmus Medical Center, Rotterdam, The Netherlands
[17]Department of Immunology, Leiden University Medical Center, Leiden, The Netherlands
[18]KU Leuven, Department of Microbiology, Immunology and Transplantation, KU Leuven University, Leuven, Belgium
[19]Histocompatibility and Immunogenetics Laboratory (HILA), Belgian Red Cross-Flanders, Mechelen, Belgium
[20]Department of Nephrology and Renal Transplantation, University Hospitals Leuven, Leuven, Belgium

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## CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

## ORCID

*Tineke Kardol-Hoefnagel* https://orcid.org/0000-0002-8736-4549
*Eric Spierings* https://orcid.org/0000-0001-9441-1019
*Wendy Swelsen* https://orcid.org/0000-0002-3637-849X
*Maarten Naesens* https://orcid.org/0000-0002-5625-0792
*Sebastiaan Heidt* https://orcid.org/0000-0002-6700-188X

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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