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SPECIAL ARTICLE

An interleukin 6-based genetic risk score strengthened with interleukin 10 polymorphisms associated with long-term kidney allograft outcomes

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Of all kidney transplants, half are still lost in the first decade after transplantation. Here, using genetics, we probed whether interleukin 6 (IL-6) could be a target in kidney transplantation to improve graft survival. Additionally, we investigated if a genetic risk score (GRS) based on *IL6* and *IL10* variants could improve prognostication of graft loss. In a prospective cohort study, DNA of 1271 donor-recipient kidney transplant pairs was analyzed for the presence of *IL6*, *IL6R*, *IL10*, *IL10RA*, and *IL10RB* variants. These polymorphisms and their GRS were then associated with 15-year death-censored allograft survival. The C|C-genotype of the *IL6* polymorphism in donor kidneys and the combined C|C-genotype in donor-recipient pairs were both associated with a reduced risk of graft loss ($p = .043$ and $p = .042$, respectively). Additionally, the GRS based on *IL6*, *IL6R*, *IL10*, *IL10RA*, and *IL10RB* variants was independently associated with the risk of graft loss (HR 1.53, 95%-CI [1.32–1.84]; $p < .001$). Notably, the GRS improved risk stratification and prediction of graft loss beyond the level of contemporary clinical markers. Our findings reveal the merits of a polygenic IL-6-based risk score strengthened with IL-10- polymorphisms for the prognostication and risk stratification of late graft failure in kidney transplantation.

KEYWORDS
interleukins, kidney transplantation, long-term graft survival, polymorphisms

Abbreviations: ABMR, antibody-mediated rejection; CKD, chronic kidney disease; cNRI, continuous net reclassification improvement; C-statistic, Harrell's concordance statistic; DGF, delayed graft function; DSA, donor-specific antibodies; GRS, genetic risk score; HLA, human leukocyte antigen; HR, hazard ratio; IDI, integrated discrimination improvement; IL-10, interleukin 10; *IL10*, interleukin 10 gene; IL-10R, interleukin 10 receptor; *IL10RA*, interleukin 10 receptor subunit alpha; *IL10RB*, interleukin 10 receptor subunit beta; IL-6, interleukin 6; *IL6*, interleukin 6 gene; IL-6R, interleukin 6 receptor; *IL6R*, interleukin 6 receptor gene; mgp130, membrane-bound glycoprotein 130; mIL-6R, membrane-bound IL-6 receptor; ROC, receiver operator characteristic; sIL-6R, soluble IL-6 receptor; SNP, single-nucleotide polymorphism; STAT3, signal transducer and activator of transcription 3.

Mariana Gaya da Costa and Bernardo Faria contributed equally.

1 | INTRODUCTION

Although kidney transplantation has considerably improved the prognosis of patients with kidney failure, long-term graft survival has hardly improved in the past decades.[1,2] Recent studies show that although ~75% of allografts from deceased donors remain vital in the first 5 years of transplantation, this percentage drops to 40%–50% 10 years post-transplantation.[3,4] The poor long-term outcomes can, in part, be rationalized by the fact that available immunosuppressants counter oligophasic T cell-mediated rejection, however, insufficiently address the onset of multiphasic alloimmunity.[4,5] Thus, to improve long-term allograft survival in kidney transplantation, new therapeutic strategies must address the additional facets of the alloimmune response to diminish the risk of late graft loss.

Overwhelming data attributes a key role to cytokines in driving overall alloimmunity. One cytokine of particular interest in this regard is the ubiquitous and pleiotropic interleukin 6 (IL-6) involved in a myriad of physiological and pathological processes—from regulating innate immunity to modulating adaptive cell- and antibody-mediated immune defenses.[6–9] Unsurprisingly, IL-6 and its receptors can cause a host of diseases and syndromes upon signaling anomalies.[10–12] There are three distinct types of IL-6 signal transduction.[6,9,13] Classic signaling applies when IL-6 heterodimerizes with the membrane-bound forms of IL-6 receptor (mIL-6R) and glycoprotein 130 (mgp130). Trans signaling, instead, occurs when IL-6 signals are transduced by soluble IL-6R (sIL-6R) in complex with mgp13.[13] Finally, trans presentation is at play when IL-6 binds mIL-6R on one cell and links up with mgp130 on another cell.[14] Due to this multitude of IL-6 signaling modes, its effects on various immune subsets can greatly differ (as described in greater detail elsewhere).[7,15–21]

Presently, we assessed the relationship between single-nucleotide polymorphisms (SNPs) in the IL-6 gene (*IL6*) and the IL-6 receptor gene (*IL6R*) with 15-year death-censored graft survival of transplanted kidneys (Figure 1). Additionally, we explored whether the combined presence of multiple variants in donor-recipient pairs, in the form of a genetic risk score (GRS), could yield more information than examining the polymorphisms individually. Notably, to strengthen the GRS we included SNPs in the IL-10 gene (*IL10*), and the genes for the subunits of the IL-10 receptor (*IL10RA* and *IL10RB*). This decision was rationalized by the fact that (i) IL-6 and IL-10 both signal through signal transducer and activator of transcription 3 (STAT3)[22] despite having opposing effects on the immune system,[20,23–25] and (ii) to investigate the relationship between the balance of pro- and anti-inflammatory IL-6 and IL-10 signaling and transplant outcomes.[26]

2 | MATERIALS AND METHODS

2.1 | Study design

Patients receiving a single kidney transplantation at the University Medical Center Groningen (UMCG) between March 1993 and February 2008 were included. Exclusion criteria consisted of perioperative, technical complications, lack of available DNA, loss of follow-up, and re-transplantation at the time of recruitment.[27–33] The primary endpoint was 15-year death-censored graft survival.

2.2 | DNA extraction and genotyping

Peripheral blood mononuclear cells from blood or splenocytes were obtained from the kidney transplant donors and their respective recipients. DNA was isolated using commercial kits. DNA samples of 1271 donor-recipient kidney transplant pairs were analyzed for variants in *IL6* (rs1800795), *IL6R* (rs2228145), *IL10* (rs1800871, rs1800896, and rs3024498), *IL10RA* (rs2229113 and rs3135932), and *IL10RB* (rs2834167). Genotyping of target SNPs was performed using the Illumina VeraCode GoldenGate Assay kit. Genotype clustering and calling were performed using BeadStudio Software. The overall genotype success rate was between 98.3% and 99.9% (Table S1). Samples with a missing call rate were excluded from subsequent analyses. Previously, we described the genotypic frequencies of the *IL6* and *IL6R* SNPs.[30] The distribution of all assessed SNP was in Hardy–Weinberg equilibrium.

2.3 | Genetic risk score

First, we dichotomized the earlier described polymorphisms based on their survival distributions of the genotypes in the Kaplan–Meier curves. Next, we constructed a GRS by assigning points for the presence or absence of the SNPs in the donors and recipients, 0 and 1 signifying absence and presence, respectively. The GRS was weighted by taking the presence of an SNP and multiplying it by the strength of the association with graft loss.[34–36] In other words, the presence of a minor allele of a polymorphism was multiplied by the beta-coefficient—also, the regression coefficient, or the logarithm of the hazard ratio regression coefficient. The beta-coefficient is negative when an SNP is associated with improved kidney transplant survival (protective) and positive when it is associated with worsened allograft survival (hazardous). The total sum of all the studied polymorphisms in both the donor and recipient determined the value of the GRS. Before generating the genetic risk score, however, we first dichotomized the polymorphisms based on their survival distributions in the Kaplan–Meier curves.

2.4 | Statistics

Statistical analysis was performed using SPSS 28.0, Stata 17.0, and R 4.2.1. Data are displayed as mean ± standard deviation (SD) for parametric variables, median [IQR] for non-parametric variables, and percentage (n [%]) for nominal data. Differences between two groups were assessed with Student's *t*-test or Mann–Whitney *U*-tests for normally and non-normally distributed variables, respectively, and χ^2-tests for categorical variables. SNPs were tested for

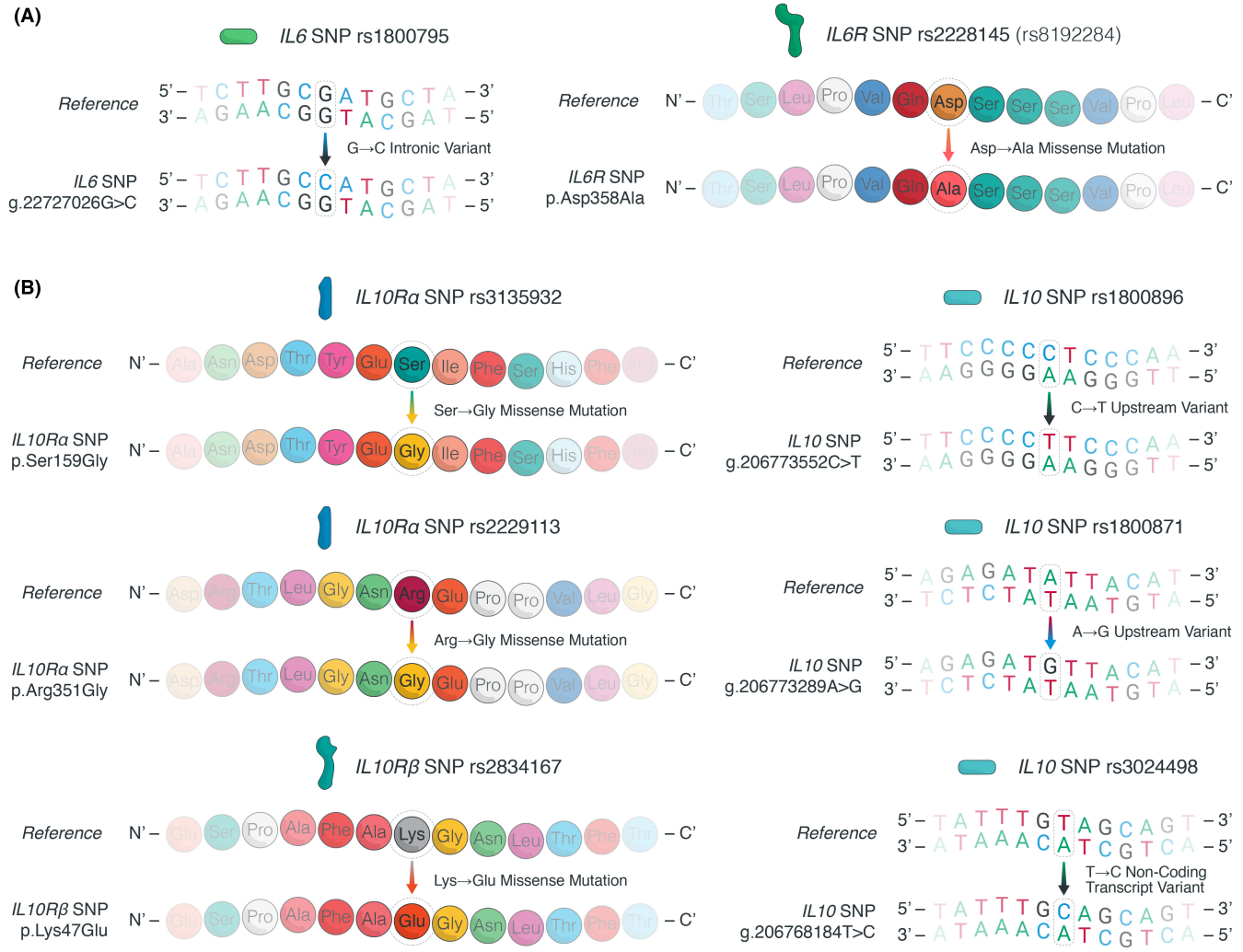

FIGURE 1 (A) IL-6-related single-nucleotide polymorphisms (SNPs) examined in the present study: rs1800795 causing a G→C nucleotide substitution resulting in an intronic variant of the IL-6 gene (*IL6*) promoter region and rs2228145 (formerly rs8192284) leading to an A→C nucleotide substitution and an Asp→Ala missense mutation of IL-6 receptor (IL6R) gene (*IL6R*). (B) IL-10-related single-nucleotide polymorphisms (SNPs) examined in the present study: rs3135932 causing an A→C nucleotide variant and a Ser→Gly missense mutation in IL-10Rα; rs2229113 leading to an A→C variant and an Arg→Gly missense mutation in IL-10 receptor alpha-unit (IL10Rα) gene (*IL10RA*); rs2834167 precipitating an A→C variant and a Lys→Glu missense mutation in IL-10 receptor beta-unit (IL10Rβ) gene (*IL10RB*); rs1800896 and rs1800871 causing C→T and A→G nucleotide substitutions, respectively, underlying independent upstream IL-10 gene (*IL10*) variants; and, rs3024498 underpinning a T→C substitution resulting in a non-coding transcript variant. Ala, alanine; Arg, arginine; Asp, aspartic acid; g., gene; Glu, glutamic acid; Gly, glycine; IL10(Rα/β), interleukin 10 (receptor alpha/beta) gene; IL6(R), interleukin 6 (receptor) gene; Lys, lysine; p., protein; Ser, serine.

associations with death-censored graft survival by Kaplan–Meier analyses with log-rank tests. Associations of SNPs with graft loss were further tested by Cox proportional-hazards regression analyses with adjustments for possible confounders.

In additional sensitivity analyses, the association of the GRS with graft loss was tested in subgroups using Cox proportional-hazards model analyses. Multivariable Cox regressions were performed to determine the association of the GRS with long-term outcomes after correcting for donor, recipient, and transplant characteristics. Furthermore, multivariable Cox regressions with stepwise forward selection were performed, which incrementally includes all the variables significantly associated with graft loss in the univariable analyses until statistical significance is lost. Finally, Harrell's C-statistic was used to assess the predictive value of the GRS when added to different reference models. When the outcome is binary, Harrell's C-statistic is equivalent to the area under the receiver operator characteristic (ROC) curve. A value of 1 equates to perfect discrimination, whereas 0.5 infers a performance comparable to random chance. We also determined the integrated discrimination improvement (IDI) and the continuous net reclassification improvement (cNRI) metrics when the GRS was included to reference models. The IDI indicates the difference between model-based probabilities for events and non-events for the models with and without the GRS. The cNRI, on the other hand, quantifies the average improvement in discrimination with the inclusion of the GRS relative to the reference model. All statistical tests were two-tailed and $p < .05$ was considered significant for all analyses.

2.5 | Ethics

The study protocol was approved by the medical ethics committee at the UMCG under file number METc 2014/077 and the study was performed in accordance with the principles of the Declaration of Helsinki.

3 | RESULTS

3.1 | Patient population and determinants of graft failure

In total, 1271 donor-recipient kidney transplant pairs were included in this study. The baseline demographic and clinical characteristics of the recipients and donors as well as the transplantation details stratified by graft loss have been listed in Table 1. Over the course of 15 years of follow-up with a mean follow-up time of 6.1 ±4.2 years, graft failure occurred in 215 of the 1271 kidney transplant recipients (16.9%). These instances of graft failure were predominantly caused by rejection ($N = 126$; including acute rejection, transplant glomerulopathy, and chronic antibody-mediated rejection). Additional causes of graft loss were the occurrence of surgical complications ($N = 33$), relapse of underlying kidney disease in recipients ($N = 16$), onset of vascular disease ($N = 12$), other causes ($N = 16$), and idiopathic graft failure ($N = 11$). In univariable analyses, recipient age, recipient blood group (AB vs. others), cyclosporin A use, corticosteroid use, donor age, donor transplant type (living donation versus donation after brain/circulatory death), donor blood group (AB vs. others), cold ischemia time, warm ischemia time, and delayed graft function (DGF) were all significantly associated with 15-year death-censored graft survival ($p < .05$).

3.2 | Common IL-6 gene variant protects against allograft loss

Using human genetics, we first investigated whether IL-6 signaling could be a suitable target in kidney transplantation to avert graft loss. Specifically, we assessed the impact of an *IL6* promoter variant (rs1800795 G→C) on long-term allograft survival by dichotomizing the donors and recipients into a C|C-genotype group and a combined C|G- and G|G-genotypes reference group. Kaplan–Meier analysis demonstrated that in the homozygous model, there was a significant survival advantage for kidney transplants from C|C-genotype donors versus donors with the combined C|G- or G|G-genotype (Figure 2A, $p = .043$). However, subgroup analyses for donor sex revealed that this association was only found in female donors (Figure 2B,C). After complete follow-up, the incidence of graft loss was 10.5% in the homozygous C|C genotype group and 18.0% in the C|G- and G|G-genotype group in the female subgroup, while no association was seen between the *IL6* polymorphism in the recipient and graft loss in the same genotype groups (Figure S1, $p = .13$). In univariable Cox regression analysis, the C|C-genotype in female kidney donors was significantly associated with death-censored graft failure (HR 0.49; 95%-CI [0.26–0.92]; $p = .026$, Table 2). This association remained significant after adjustment for potential confounders in multivariable analyses (Table 2), including donor, recipient, and transplant characteristics (models 2–4, respectively). Herein, we included the donor, recipient, and transplant characteristics separately to prevent overfitting the data. Subsequently, we determined whether the Asp358Ala variant in the *IL6R* (rs2228145 A→C; previously rs8192284) was associated with death-censored graft loss. We dichotomized the genotypes into a homozygous C|C-genotype group and a matched A|A- and A|C-genotype group. Kaplan Meyer analysis revealed no significant difference in 15-year death-censored graft survival between the *IL6R* genotypes among the donors (Figure S1, $p = .85$) or the recipients (Figure S1, $p = .82$).

Next, we investigated if donor-recipient mismatches for the *IL6* polymorphisms were associated with the risk of death-censored graft loss. To do so, kidney transplant pairs were divided into three groups: (i) neither the donor nor the recipient having the C|C-genotype, (ii) either the donor or the recipient having the C|C-genotype, or (iii) both the donor and the recipient having the C|C-genotype. No significant differences were found in any bassline demographic and clinical characteristics between the donor-recipient pairs stratified by the *IL6* polymorphism (Table S2). Kaplan–Meier survival analysis revealed a significant difference in death-censored graft survival among the three groups (Figure 2D, $p = .042$). Donor-recipient pairs with a combined C|C-genotype of the *IL6* polymorphism had the best outcome. The cumulative 15-year death-censored kidney allograft survival was 92.6% in the combined donor-recipient C|C-genotype group, 78.2% for donor-recipient pairs where either the donor or the recipient had the C|C-genotype, and 71.0% for donor-recipient pairs without the C|C-genotype, respectively. Subgroup analysis for sex did not change these results. In univariable Cox regression analysis, the combined C|C-genotype in donor-recipient pairs was significantly associated with death-censored graft failure with a hazard ratio of 0.70 (95%-CI [0.53–0.94]; $p = .016$). Furthermore, when the multivariable analysis was performed to adjust for potential confounders, the combined C|C-genotype of the *IL6* polymorphism in donor-recipient pairs remained significantly associated with death-censored graft survival (Table 2). These results indicate that a common functional variant in the *IL6* associated with a lower incidence of death-censored graft loss in the context of kidney transplantation. To reinforce this monogenic signature in kidney transplantation, we decided to fortify this association with the inclusion of additional gene variants that could impact graft survival. Although there were several candidate genes, we focused on polymorphisms in *IL10*, *IL10RA*, and *IL10RB*, hypothesizing that this would create a more holistic polygenic risk score due to the overlapping signaling machinery used by IL-6 and IL-10 and the importance of the stoichiometry between pro- and anti-inflammatory signals in determining net outcomes of individual cytokine signals.[22,26]

TABLE 1 Baseline demographic and clinical characteristics of kidney transplant donors and recipients overall and stratified by graft loss

	All patients (*N* = 1271)	Functioning graft (*N* = 1056)	Graft loss (*N* = 215)	*p*-value[a]	Hazard ratio	*p*-value[b]
Recipient						
Female sex, *N* (%)	532 (41.9)	449 (42.5)	83 (38.6)	.29		.21
Age, years (SD)	47.9 (±13.5)	48.5 (±13.4)	45.0 (±13.2)	**<.001**	0.99	**.027**
Dialysis vintage, weeks [IQR]	172 [91–263]	174 [87–261]	168 [109–270]	.15		.10
Blood group						
Type O, *N* (%)	567 (44.6)	474 (44.9)	93 (43.3)	**.004**	0.46	**.002**
Type A, *N* (%)	536 (42.2)	448 (42.4)	88 (40.9)		0.46	**.002**
Type B, *N* (%)	113 (8.9)	98 (9.3)	15 (7.0)		0.35	**.002**
Type AB, *N* (%)	55 (4.3)	36 (3.4)	19 (8.8)		Ref.	**.008**
Primary kidney disease				.32		.45
Glomerulonephritis, *N* (%)	340 (26.8)	271 (25.7)	69 (32.1)			
Polycystic disease, *N* (%)	208 (16.4)	187 (17.7)	21 (9.8)			
Vascular disease, *N* (%)	145 (11.4)	122 (11.6)	23 (10.7)			
Pyelonephritis, *N* (%)	148 (11.6)	120 (11.4)	28 (13.0)			
Diabetes, *N* (%)	51 (4.0)	44 (4.2)	7 (3.3)			
Chronic, *N* (%)	168 (13.2)	134 (12.7)	34 (15.9)			
Other, *N* (%)	211 (16.6)	178 (16.9)	33 (15.3)			
Immunosuppression						
Anti-CD3 mAb, *N* (%)	19 (1.5)	14 (1.3)	5 (2.3)	.27		.51
ATG, *N* (%)	103 (8.1)	79 (7.5)	24 (11.2)	.07		.14
Azathioprine, *N* (%)	72 (5.7)	53 (5.0)	19 (8.8)	**.027**		.29
Corticosteroids, *N* (%)	1201 (94.5)	1002 (94.9)	199 (92.6)	.17	0.51	**.01**
Cyclosporin A, *N* (%)	1085 (85.4)	911 (86.3)	174 (80.9)	**.044**	0.66	**.016**
Interleukin-2 RA, *N* (%)	199 (15.7)	163 (15.4)	36 (16.7)	.63		.12
MMF, *N* (%)	907 (71.4)	775 (73.4)	132 (61.4)	**<.001**		.06
Sirolimus, *N* (%)	38 (3.0)	33 (3.1)	5 (2.3)	.53		.54
Tacrolimus, *N* (%)	97 (7.6)	77 (7.3)	20 (9.3)	.31		.39
Donor						
Female sex, *N* (%)	626 (49.3)	521 (49.3)	105 (48.8)	.89		.96
Age, years (SD)	44.4 (±14.4)	44.1 (±14.6)	46.1 (±13.4)	**.044**	1.02	**<.001**
Donor type						
Living, *N* (%)	282 (22.2)	257 (24.3)	25 (11.6)	**<.001**	Ref.	**.002**
DBD, *N* (%)	787 (61.9)	642 (60.8)	145 (67.4)		1.94	
DCD, *N* (%)	202 (15.9)	157 (14.9)	45 (20.9)			
Blood group						
Type O, *N* (%)	642 (50.6)	541 (51.3)	101 (47.2)	**.033**	0.39	**.004**
Type A, *N* (%)	502 (39.6)	414 (39.3)	88 (41.1)		0.42	**.01**
Type B, *N* (%)	97 (7.6)	82 (7.8)	15 (7.0)		0.36	**.012**
Type AB, *N* (%)	27 (2.1)	17 (1.6)	10 (4.7)		Ref.	**.035**
Transplantation						
Highest PRA, % (SD)	10.1 (±23.6)	9.98 (±23.7)	10.9 (±25.0)	.54		.75
Total HLA mismatches [IQR]	2 [1–3]	2 [1–3]	2 [1–3]	.48		.11

(Continues)

TABLE 1 (Continued)

	All patients (*N* = 1271)	Functioning graft (*N* = 1056)	Graft loss (*N* = 215)	*p*-value[a]	Hazard ratio	*p*-value[b]
CIT, h [IQR]	17.7 [11–23]	17.0 [9–23]	20.0 [15–25]	**<.001**	1.03	**.001**
WIT, min [IQR]	37.0 [31–45]	37.0 [30–45]	38.0 [32–45]	.12	1.02	**.003**
DGF, *N* (%)	415 (32.7)	289 (27.4)	126 (58.6)	**<.001**	3.79	**<.001**

Note: The characteristics of all donor and recipient kidney transplant pairs as well as subgroup analyses for graft loss at 15 years of follow-up. Data are displayed as the total number of patients with percentages for nominal data (*N* [%]), median (IQR) for non-parametric variables, and mean ± SD for parametric variables. Bolded *p*-values indicate statistical significance ($p < .05$).

Abbreviations: ATG, anti-thymocyte globulin; CD3, cluster of differentiation 3; CIT, cold ischemia time; DBD, donation after brain death; DCD, donation after circulatory death; DGF, delayed graft function; HLA, human leukocyte antigen; IQR, interquartile range; mAb, monoclonal antibody; MMF, mycophenolate mofetil; N, number; PRA, panel-reactive antibody; RA, receptor antagonist; Ref, reference; SD, standard deviation; WIT, warm ischemia time.

[a]*p*-value, differences in baseline demographics between the groups, tested by χ^2 tests for categorical variables, and Mann–Whitney *U* or Student's *t*-tests for continuous variables.

[b]*p*-value, univariable Cox regression analyses for 15-year death-censored graft survival.

3.3 | Polymorphisms in IL-10 and its receptors do not individually impact graft survival

First, we studied the association between common genetic variants in IL-10 and its receptor with death-censored graft survival. Particularly, we assessed rs3135932 leading to a Ser → Gly missense mutation in IL-10Rα, rs2229113 precipitating an Arg → Gly missense mutation in IL-10Rα, rs2834167 causing a Lys → Glu missense mutation in IL-10Rβ, rs1800896, and rs1800871 causing C → T and A → G nucleotide substitutions, respectively, underlying independent upstream IL-10 gene variants, and rs3024498 underpinning a T → C substitution resulting in a non-coding transcript variant (Figure 1B). The genotype frequencies of the *IL10* and *IL10R* SNPs in the donors and recipients, as well as the overall 1000 genomes reference group and European subgroup therein[37–39] have been provided in Table S3. Using Kaplan–Meier survival analyses, we next investigated the impact of the individual genetic variants in IL-10 and its receptor on long-term kidney transplant survival (Table 3). Notably, we did not observe a significant difference in 15-year death-censored graft loss for any of the *IL10* and *IL10R* SNPs in the transplant donors or recipient, demonstrating that the gene variants in IL-10 and its receptor did not individually impact graft survival in the context of kidney transplantation.

3.4 | A genetic risk score based on IL6 and IL10 polymorphisms associated with long-term allograft survival

To strengthen the monogenic IL-6 association with graft loss, we developed a genetic risk score (GRS) fortifying the IL-6 polymorphisms with SNPs in the IL-10 cytokine and receptor (Table S4). In brief, this GRS assesses at the presence of 8 different polymorphisms across 5 genes in the kidney transplant donor and recipient. The score is weighted by the presence of an SNP according to its hazard ratio, resulting in a negative score for protective polymorphisms and a positive one for hazardous ones. When the net GRS is above zero, this indicates there are more hazardous SNPs present in a donor-recipient pair, while a net GRS below zero indicates the presence of more protective SNPs in a donor-recipient pair. To assess the clinical application of the GRS, we studied its predictive value in greater detail. First, we found that the GRS was significantly associated with death-censored graft loss (HR 1.49, 95%-CI [1.27–1.76]; $p < .001$ per SD increase). Upon dividing the GRS in tertiles, the Kaplan–Meier curves showed increasing rates of graft loss with increasing GRS values in the donor-recipient pairs (Figure 3A; log-rank $p < .001$). Specifically, the 15-year graft survival in the lowest tertile was 83.6%, 70.0% in the middle tertile, and 66.3% in the highest tertile. The association of the GRS with long-term graft survival was additionally consistent in the subgroup analyses (Figure 3B). The confidence intervals of the hazard ratio in the subgroups displayed substantial overlap with the hazard ratio in the entire cohort, showing the consistency of the relationship between the GRS and graft loss across the subgroups. Next, multivariable regression analyses were performed to adjust for donor, recipient, and transplant characteristics (models 2–4, respectively; Table 4), the GRS retaining the significant association with graft loss in all models. Finally, we performed a multivariable analysis with a stepwise forward selection procedure. In the final model, the GRS, donor and recipient age, recipient blood type, and DGF were all included (Table 5). Altogether, these analyses support an independent association of the GRS with long-term graft survival following kidney transplantation based on IL-6 SNPs fortified with IL-10-specific polymorphisms.

3.5 | Predictive value of the IL-6/IL-10 genetic risk score for graft loss

To test the discrimination performance of the various models, we calculated Harrell's concordance statistic (C-statistic). The GRS alone had a C-statistic of 0.61 (95%-CI [0.56–0.66]; $p < .001$). When the GRS was added to models containing donor, recipient, and transplant

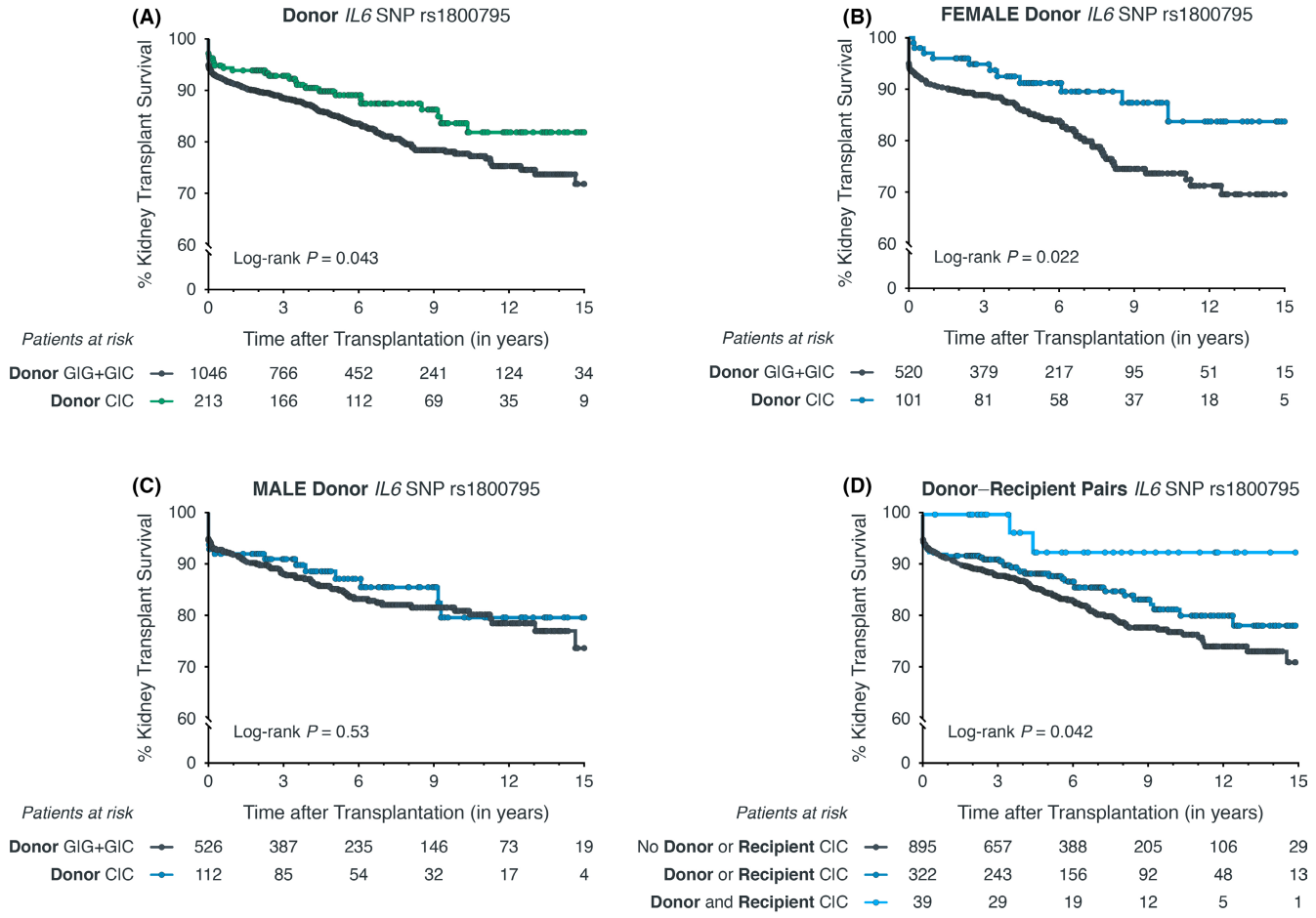

FIGURE 2 Kaplan-Meier survival curves of 15-year death-censored kidney allografts survival stratified by *IL6* rs1800795 genotypes in the donors and donor-recipient transplant pairs. (A) Cumulative death-censored survival of kidney allografts based on the *IL6* rs1800795 single nucleotide polymorphism (SNP) genotypes in allograft donors. The homozygous C|C-genotype (homozygous model; green line) was compared to the heterozygous C|G-genotype combined with the refence homozygous G|G-genotype (black line). (B and C) Cumulative death-censored survival of kidney allografts among transplant recipients with (B) female and (C) male allograft donors based on the *IL6* rs1800795 single nucleotide polymorphism (SNP) genotypes. The homozygous C|C-genotype (homozygous model; blue line) was compared to the heterozygous C|G-genotype combined with the refence homozygous G|G-genotype (black line). (D) Cumulative death-censored survival of kidney allografts based on the donor-recipient paired genotypes of the *IL6* rs1800795 SNP, comparing (i) pairs with neither donor nor recipient C|C-genotype presence (black line), (ii) presence of the C|C-genotype in either donor or recipient (dark blue line), with (iii) C|C-genotype presence in both the donor and recipient (light blue line). Data represent death-censored survival curves. *p*-values were calculated using log-rank tests. *IL6*, interleukin 6 gene.

characteristics (models 2–4, respectively), each model's discriminative accuracy for graft loss significantly improved (Table 6). The highest discriminative accuracy was reached when only significant determinants of graft survival, including pre- and posttransplant variables, were added (C-statistic 0.69, 95%-CI [0.65–0.74]; $p < .001$; model 5). Furthermore, the predictive value of the models significantly improved as judged by the integrated discrimination improvement index (IDI). The addition of the GRS to the model that contained only significant determinants of graft survival resulted in an IDI value of 3.1%, confirming the GRS substantially improved the prediction of graft loss. The continuous net reclassification improvement (cNRI) analysis finally revealed that the addition of the GRS to the previously described models significantly improved the classification (Table 6). Once again, the highest cNRI was seen for the model that included only significant determinants of graft survival (cNRI 45.2%, 95%-CI [28%–60%]; $p < .001$; model 5) Altogether, these analyses infer that the IL-6/IL-10-based GRS substantially improved risk prediction for graft loss beyond the contemporary markers used in the clinic.

4 | DISCUSSION

An improved understanding of the immunological pathways that drive graft loss is vital to create tailored therapies in transplantation—both to treat elicited alloimmune responses and to prevent the onset thereof.[40,41] Herein, human genetic studies provide a complementary model permitting the assessment of causal mechanisms for target validation.[42,43] More importantly, therapeutic targets supported by human genetic evidence in disease association studies have a two-fold increased chance of leading to approved drugs.[44,45] Our

TABLE 2 Associations of *IL6* rs1800795 polymorphism and 15-year death-censored graft loss following kidney transplantation

	In female donor			Donor-recipient pairs		
IL6 SNP (rs1800795)	Hazard ratio	95% CI	*p*-value	Hazard ratio	95% CI	*p*-value
Model 1	0.49	0.26–0.92	.026	0.70	0.53–0.94	.016
Model 2	0.49[a]	0.26–0.92	.026	0.69	0.52–0.92	.012
Model 3	0.53	0.28–0.99	.047	0.72	0.54–0.97	.028
Model 4	0.35	0.17–0.72	.005	0.65	0.48–0.88	.006

Note: Uni- and multivariable Cox regression analyses were performed for 15-year death-censored graft survival.Model 1: crude model.

Model 2: adjusted for model 1 plus donor characteristics—donor age, donor sex, donor blood type, and donor origin.

Model 3: adjusted for model 1 plus recipient characteristics—recipient age, recipient sex, recipient blood type, and dialysis vintage.

Model 4: adjusted for model 1 plus transplant characteristics—cold and warm ischemia times, total HLA-mismatches, and the occurrence of delayed graft function (DGF).

Data are displayed as the hazard ratios with 95% confidence intervals and *p*-values of the Cox proportional-hazards analyses.

[a]For the *IL6* SNP in the donor, model 2 could not be adjusted for donor sex since this was a subgroup analysis for female donors.

TABLE 3 Associations of IL-10 cytokine and receptor polymorphisms and 15-year death-censored graft loss following kidney transplantation

			Donor		Recipient	
Gene	SNP	Genotype	Graft survival % (*N* at risk)	*p*-value	Graft survival % (*N* at risk)	*p*-value
IL10RA	rs3135932	A|A	82.7% (155/895)	.47	83.7% (138/848)	.20
		A|G	84.6% (55/357)		81.4% (74/397)	
		G|G	92.3% (1/13)		95.7% (1/23)	
IL10RA	rs2229113	A|A	82.3% (22/124)	.87	84.8% (17/112)	.73
		A|G	82.9% (103/601)		83.5% (91/550)	
		G|G	84.1% (85/534)		82.7% (105/608)	
IL10RB	rs2834167	A|A	82.9% (120/702)	.19	83.3% (120/719)	.39
		A|G	84.4% (79/506)		83.6% (75/458)	
		G|G	77.0% (14/61)		80.6% (18/93)	
IL10	rs1800896	C|C	83.6% (48/292)	.15	83.0% (53/312)	.56
		C|T	81.5% (124/670)		82.5% (113/646)	
		T|T	86.7% (41/308)		84.8% (47/309)	
IL10	rs1800871	A|A	88.1% (8/67)	.47	80.0% (15/75)	.35
		A|G	82.6% (75/430)		81.7% (82/449)	
		G|G	82.8% (130/756)		84.4% (115/735)	
IL10	rs3024498	T|T	82.6% (108/622)	.79	83.2% (110/655)	.85
		T|C	83.7% (87/534)		83.7% (86/526)	
		C|C	84.8% (14/92)		80.7% (17/88)	

Note: A total of 1271 donor-recipient kidney transplantation pairs were analyzed for the presence of genetic variants in the interleukin 10 cytokine (IL-10) and receptor (IL-10Rα, IL-10Rβ) genes. Data are displayed as the 15-year, death-censored graft survival times per SNP subgroup and p-values of the log-rank tests. Additional allelic variants of rs2229113 and rs2834167 (A→T for both loci) have also been described.

Abbreviations: CI, confidence interval; *IL10*, interleukin 10; *IL10RA*, interleukin 10 receptor alpha gene; *IL10RB*, interleukin 10 receptor beta gene; *N*, number; *SNP*, single nucleotide polymorphism.

key finding here is the association of a functional *IL6* polymorphism in the transplant donor with long-term graft survival following kidney transplantation. Particularly, we found that kidney transplant pairs with a combined C|C-genotype of the *IL6* rs1800795 SNP in donors and recipients had the lowest risk of graft failure. Elaborating on these findings, we developed a GRS incorporating IL-6 and

IL-10-related SNPs. This IL-6/IL-10 GRS proved to be a major determinant of late graft failure and refined the risk prediction for graft loss beyond what current clinical risk factors permit. Overall, our results offer genetic evidence for a key role of IL-6 in graft survival after kidney transplantation, encouraging clinical trials exploring the use of IL-6-specific inhibitors in this context.

TABLE 4 Associations of IL-6/-10 donor-recipient pair genetic risk score and 15-year death-censored graft loss following kidney transplantation

	IL-6/IL-10 genetic risk score		
	Hazard ratio (per SD)	95% CI	*p*-value
Model 1	1.494	1.271–1.756	<.001
Model 2	1.491	1.263–1.761	<.001
Model 3	1.457	1.233–1.722	<.001
Model 4	1.621	1.363–1.928	<.001

Note: Uni- and multivariable Cox regression analyses were performed for the association between the genetic risk score (GRS) and 15-year death-censored graft survival.

Model 1: crude model.

Model 2: adjusted for model 1 plus donor characteristics—donor age, donor sex, donor blood type, and donor origin.

Model 3: adjusted for model 1 plus recipient characteristics—recipient age, recipient sex, recipient blood type, and dialysis vintage.

Model 4: adjusted for model 1 plus transplant characteristics—cold and warm ischemia times, total HLA-mismatches, and the occurrence of delayed graft function (DGF).

Data are displayed as the hazard ratios with 95% confidence intervals and *p*-values of the Cox proportional-hazards analyses.

To our knowledge, our study is the first to show that the *IL6* rs1800795 polymorphism in donors and donor-recipient kidney transplant pairs associated with 15-year death-censored graft survival after kidney transplantation.[46–48] Previously, we reported on the association between the same *IL6* rs1800795 polymorphism in kidney donors and the risk of biopsy-proven acute rejection.[30] However, the association between the *IL6* rs1800795 SNP in donors and BPAR was only found in male donors after subgroup analyses stratified for donor sex.[30] The current association between the *IL6* rs1800795 SNP in donors and graft loss, in contrast, was predominantly found in female donors (data not shown). This sex-related difference might also explain the conflicting findings presented in previous studies.[47,49–54] Our study highlights that sex should indeed be considered in transplantation and for cytokine-targeted therapies. Furthermore, considering the sex-related differences in the impact of this *IL6* variant, stratification by sex might be helpful in the interpretation of clinical trials with IL-6 inhibitors.

Previous studies on the functional consequences of this variant have expanded our understanding of the molecular mechanisms through which this *IL6* variant could impact transplant outcomes. In 1998, the first description of the *IL6* rs1800795 polymorphism was accompanied by the finding that the C-allele decreased IL-6 production relative to the G-allele.[49] Similarly, Bamoulid and colleagues showed decreased serum IL-6 and CRP concentrations

TABLE 5 Multivariable analyses for the 15-year death-censored graft survival after kidney transplantation

Variables	Hazard ratio	*p*-value
IL-6/−10 genetic risk score (per SD)	1.525 (1.306–1.817)	<.001
Delayed graft function (yes vs. no)	4.510 (3.371–6.035)	<.001
Recipient blood type (AB vs. other)		.001
AB vs. *O*	0.381 (0.229–0.635)	<.001
AB vs. *A*	0.389 (0.233–0.649)	<.001
AB vs. *B*	0.294 (0.144–0.601)	.001
Recipient age (in years)	0.982 (0.972–0.993)	.001
Donor age (in years)	1.014 (1.004–1.025)	.006
Warm ischemia time (in minutes)		.06
Corticosteroids		.06
Donor type (living vs. deceased)		.16
Cold ischemia time (in hours)		.30
Cyclosporin A		.34
Donor blood type (AB vs. other)		.86

Note: Multivariable Cox proportional-hazards regression analyses for 15-year death-censored graft survival using a forward selection method. Variables with $p < 0.05$ in univariable analyses were included in the multivariable regression analyses. In the final model, the IL-6/-10 donor-recipient pair genetic risk score, the occurrence of delayed graft function, recipient age, recipient blood type, and donor age were statistically significant, whereas donor transplant type, donor blood type, warm ischemia time, cold ischemia time, use of corticosteroids, and use of cyclosporin A were not. Data are presented as hazard ratios with 95% confidence intervals and *p*-values of the Cox proportional-hazards analyses.

Abbreviations: CI, confidence interval; SD, standard deviation; vs, versus.

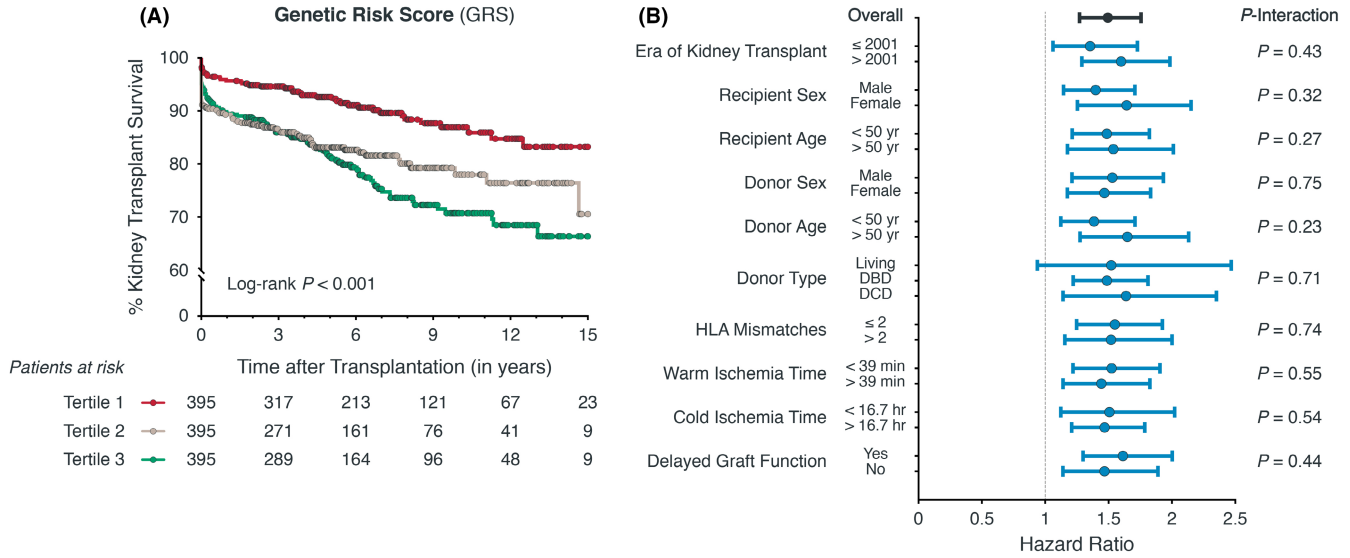

FIGURE 3 Kaplan-Meier survival curves of 15-year death-censored kidney allograft survival stratified by IL-6/-10 genetic risk score tertiles in donor-recipient kidney transplant pairs. (A) Cumulative death-censored graft survival of kidney transplants based on tertiles of the interleukin 6 (IL-6)/interleukin 10 (IL-10) genetic risk score (GRS). The log-rank test was used to compare the incidence of graft loss between the groups. (B) Forest plot of the hazard ratio of the IL-6/-10 with 15-year death-censored graft loss in our kidney transplant population and relevant subgroups. Data represent the hazard ratios with 95% confidence intervals of the GRS for 15-year death-censored graft survival in each population. The P-interaction represents the P-value for the interaction term between the GRS and the subgroup characteristic to identify potential modifiers in the Cox regression models. DBD, donation after brain death; DCD, donation after circulatory death; GRS, genetic risk score; HLA, human leukocyte antigen; hr, hour(s); min, minute(s); yr, year(s).

TABLE 6 Additive value the IL-6/IL-10 genetic risk score for the prediction of death-censored allograft survival

	C-statistic		Change		IDI		cNRI	
	Without the GRS	With the GRS	(95% CI)[a]	*p*-value[a]	(%)	*p*-value[b]	(%)	*p*-value[c]
Model 1	0.500 (N/A)	0.611 (0.56–0.66)	N/A	N/A	N/A	N/A	N/A	N/A
Model 2	0.592 (0.55–0.64)	0.642 (0.59–0.69)	0.050 (0.01–0.09)	.018	2.33 (1.4–3.3)	<.001	34.7 (19–51)	<.001
Model 3	0.583 (0.53–0.63)	0.633 (0.59–0.68)	0.050 (0.01–0.09)	.025	2.2 (1.1–3.2)	<.001	37.5 (22–54)	<.001
Model 4	0.630 (0.58–0.68)	0.682 (0.63–0.73)	0.053 (0.01–0.09)	.010	3.25 (2.1–4.5)	<.001	45.2 (29–61)	<.001
Model 5	0.655 (0.61–0.70)	0.692 (0.65–0.74)	0.037 (0.01–0.07)	.014	3.06 (1.9–4.2)	<.001	45.2 (28–60)	<.001

Note: Data are presented as Harrell's concordance statistic (C-statistic), integrated discrimination improvement (IDI) and continuous net reclassification improvement (cNRI) with 95% confidence interval (CI).

Model 1: crude model.

Model 2: adjusted for model 1 plus donor characteristics—donor age, donor sex, donor blood type, and donor origin.

Model 3: adjusted for model 1 plus recipient characteristics—recipient age, recipient sex, recipient blood type, and dialysis vintage.

Model 4: adjusted for model 1 plus transplant characteristics—cold and warm ischemia times, total HLA-mismatches, and the occurrence of delayed graft function (DGF).

Model 5: adjusted for model 1 plus all other significant determinants of graft survival.

Abbreviations: CI, confidence interval; cNRI, continuous net reclassification improvement; GRS, genetic risk score; IDI, integrated discrimination improvement; N/A, not applicable; w/, with; w/o, without.

[a]Change in C-statistics when the model without the genetic risk score (GRS) was compared to the model that included GRS, with the corresponding *p*-value.

[b]*p*-value for the IDI when adding the genetic risk score (GRS) to the model.

[c]*p*-value for the cNRI when adding the genetic risk score (GRS) to the model.

in kidney transplant recipients with the C-allele, with the lowest circulating IL-6 levels in individuals with the C|C-genotype.[47] In other studies, however, the serum IL-6 concentrations in C|C-homozygotes were found to be elevated relative to the G-allele genotypes.[55–57] A potential explanation for these seemingly conflicting findings was provided by a recent study, that revealed discordant effects of the rs1800795 polymorphism on IL-6 production by diverse cell types.[58] Additionally, as pointed out by Brull and colleagues, genotypes of this SNP are marked by different kinetic profiles of IL-6 release, adding an additional layer of complexity to determining its functional outcomes.[59] Altogether, we speculate that the *IL6* rs1800795 SNP indeed impacts the risk of disease by modifying IL-6 levels—notably, however, an impact that is dependent on the cellular origin of IL-6 and the different kinetic profiles at play.

In the context of kidney transplantation, clazakizumab is a humanized IL-6-directed monoclonal antibody under evaluation for antibody-mediated rejection (ABMR).[60] In a phase 2 trial with 20 transplant recipients with chronic active ABMR, recipients were randomized for clazakizumab or placebo, followed by an open-label extension where all participants received clazakizumab.[61] Although clazakizumab treatment was associated with increased infection rates and diverticular disease complications, there were significant reductions in donor-specific antibodies (DSA) in clazakizumab-treated recipients and attenuation of the activity and progression of ABMR. A subsequent open-label trial with 10 transplant recipients with chronic ABMR similarly showed that clazakizumab treatment decreased DSAs and supported kidney function stabilization, without notably elevated infection rates.[62] Finally, a phase 3 trial for clazakizumab in ABMR patients is ongoing (NCT03744910). Interestingly, mechanistically the various IL-6-specific therapies seemingly boost endogenous immunoregulatory immune subsets such as $CD4^+FoxP3^+$ regulatory T cells (T_{regs})[63,64] —IL-6 known to physiologically diminish quantity and quality of T_{regs}.[17,65] Indeed, in a desensitization study of highly sensitized patients awaiting transplantation with clazakizumab, T_{reg} numbers were significantly increased, pointing to the anti-inflammatory synergy between IL-6 therapy and T_{regs}.[66]

Overall, our present findings provide key considerations for IL-6 targeting in kidney transplantation, including on the site of action, sex differences, and patient selection. Since we found that an *IL6* polymorphism in donors, but not recipients, is associated with the risk of graft loss, targeting intragraft IL-6 production over circulating IL-6 in the recipient could help improve transplant outcomes. A growing body of evidence demonstrates the importance of donor characteristics in transplant outcomes. Kidney transplants from the same donor have been shown to have similar allograft outcome, despite being transplanted into different recipients.[67,68] In accordance, we have previously found that donor genetics have a bigger impact on allograft survival than recipient genetics.[27,29–32] In this regard, our GRS could be a complementary tool to identify transplant donor-recipient pairs with an elevated risk of long-term graft failure or to stratify donor-recipient pairs with the greatest expected gain from IL-6 blockade. Our results here should be considered in the light of certain limitations. Since our study is prospective but observational in nature, the associations presented here are expected to be causal, although we cannot rule out the presence of linked SNPs in neighboring genomic regions of the assessed SNPs. Furthermore, we solely analyzed individual polymorphisms for *IL6*, *IL6R*, *IL10*, *IL10RA*, and *IL10RB* and not haplotypes thereof. Due to a lack of plasma samples, we also could not correlate the various SNP genotypes to circulating levels of IL-6 and IL-10, and due to the lack of a standardized protocol and detailed histopathological analysis of lost grafts, we could not investigate if the association between the IL6 variant and 15-year death-censored graft survival was related to specific causes of graft loss. Future studies are warranted in this regard to assess pathological differences between genotypic groups, functional levels of IL-6 and IL-10 in the allograft and circulation, and circulating donor-specific antibodies associated with humoral immunity, but also to externally validate our findings in a separate cohort. Finally, considering the discriminative performance of our GRS, additional polymorphisms could be incorporated to improve its predictive performance. Notable strengths of our study include the cohort size, robust statistical analyses, its long follow-up time of 15 years and clinically relevant endpoint, namely death-censored graft loss.

In conclusion, the *IL6* rs1800795 variant in kidney transplant donors and donor-recipient transplant pairs associated with the long-term risk of graft failure following transplantation, insinuating a potentially central role for IL-6 in driving chronic alloimmunity. Systemic trials of therapeutics targeting the IL-6 pathway and its downstream effectors in the context of kidney transplantation are warranted to identify the circumstances wherein IL-6 inhibitors can be most effective and they can synergize with endogenous immunoregulatory subsets.

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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*.

DATA AVAILABILITY STATEMENT

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

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