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Association of Carboxypeptidase B2 Gene Polymorphisms With Graft Loss in Kidney Transplantation

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Background. Plasma carboxypeptidase B2 (CPB2) is an enzyme that regulates protein activities by cleaving C-terminal amino acids. With its anti-inflammatory and antifibrinolytic properties, CPB2 can have protective or harmful effects on disease. We investigated the impact of CPB2 on long-term outcomes after kidney transplantation. **Methods.** This observational cohort study involved 1271 renal transplant pairs from the University Medical Center Groningen in The Netherlands and analyzed 4 CPB2 gene (*CPB2*) polymorphisms (rs2146881, rs3742264, rs1926447, and rs3818477) and 2 complement polymorphisms (rs2230199 and rs17611) in both donors and recipients, in relation to 15-y allograft survival. **Results.** The CPB2 rs3742264 polymorphism in the donor was associated with a reduced risk of graft loss after kidney transplantation (hazard ratio: 0.71 for the *CPB2147T* variant; 95% confidence interval, 0.55-0.93; $P = 0.014$). This association remained significant after comprehensive adjustments. However, the protective effect of the *CPB2147T* variant in the donor could be mitigated by the hazardous effect of gain-of-function complement polymorphisms. Additionally, we compiled a genetic risk score based on the 4 CPB2 variants in the recipients and donors. This genetic risk score was independently associated with long-term allograft survival and substantially improved risk prediction for graft loss beyond currently used clinical predictors. **Conclusions.** Kidney allografts possessing the *CPB2147T* variant have a lower risk of graft loss following kidney transplantation. Moreover, our findings suggest that CPB2 might protect against graft loss by inactivating complement anaphylatoxins.

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INTRODUCTION

The innate immune system and coagulation cascades play a significant role in graft injury and transplant outcomes.[1,2] Furthermore, the interaction between these systems is a potential therapeutic target in transplantation.[3] As enzymes with both antifibrinolytic and anti-inflammatory qualities, carboxypeptidases are a target of interest within this scope.

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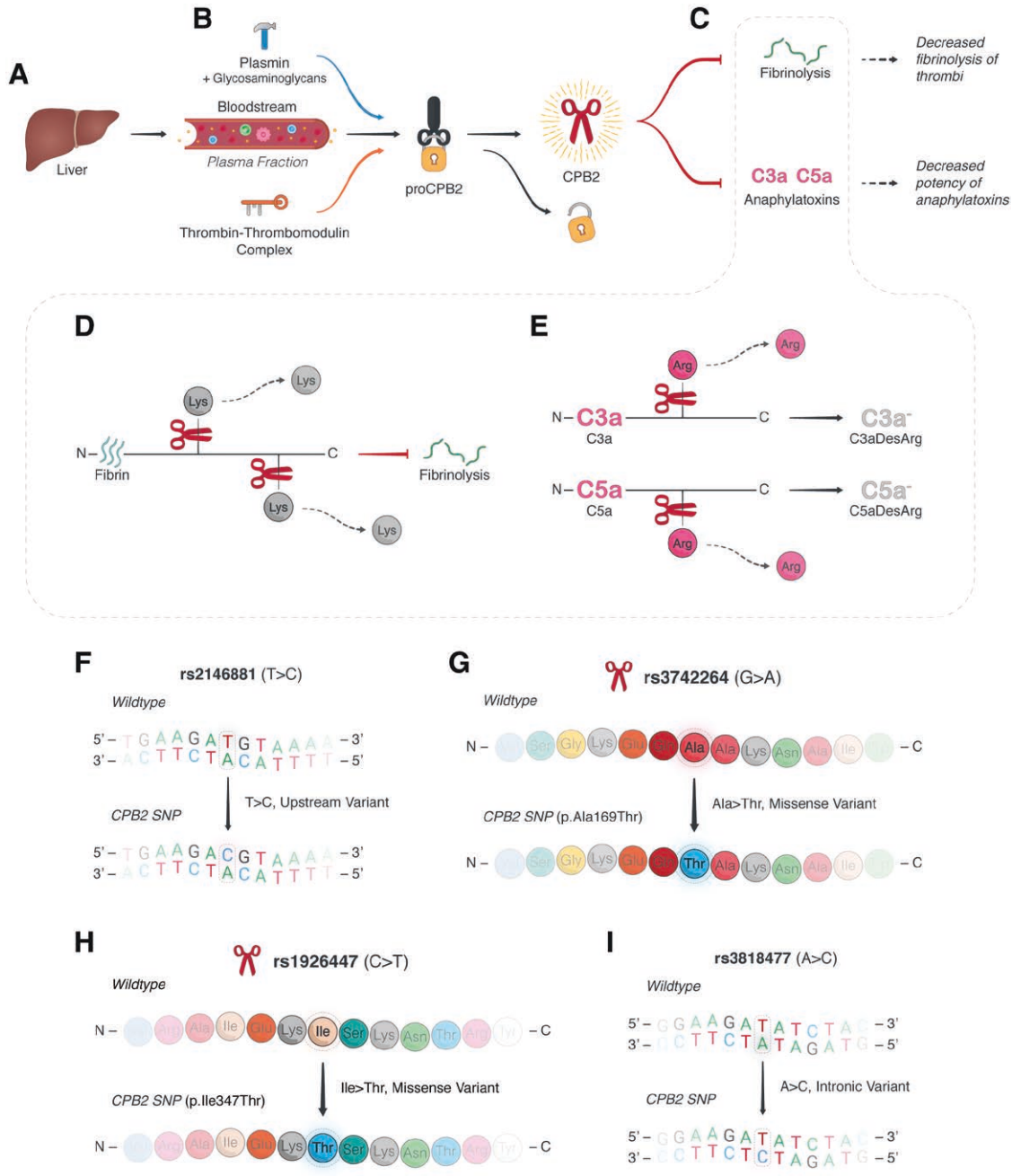


FIGURE 1. Illustration of the CPB2 pathway and examined *CPB2*-related polymorphisms. A, A precursor form of CPB2 (proCPB2) is produced in the liver and released into the plasma fraction of the blood. B, There, it can interact with plasmin bound to glycosaminoglycans and thrombin-thrombomodulin complex to gain its enzymatic and carboxypeptidase activity as mature CPB2. C–E, The main effects of CPB2 are exerted on fibrinolysis and anaphylatoxin (C). D, The effect on fibrinolysis is mediated through fibrin by CPB2-dependent removal of C-terminal lysine and arginine residues, thus thrombus resorption by fibrinolytic mechanisms. E, The neutralization of anaphylatoxins such as C3a and C5a is mediated by C-terminal lysis of arginine residues, creating C3aDesArg and C5aDesArg variants with decreased inflammatory potency, resulting in anti-inflammatory effects. F–I, In this study, we assessed the associations of 4 SNPs in kidney allograft donors and recipients on graft survival. These SNPs were rs2146881 (G>A) (F), rs3742264 (p.Ala169Thr) (G), rs1926447 (p.Ile347Thr) (H), and rs3818477 (A>C) (I). C3a, complement 3a; C5a, complement 5a; fbrn, fibrin; flysis, fibrinolysis; (pro)CPB2, (pro)carboxypeptidase B2; SNP, single-nucleotide polymorphism.

Carboxypeptidases are a family of zinc-containing proteolytic enzymes regulate the activities of biologically active proteins by cleaving C terminus amino acids.[4] They can be soluble or membrane-bound, with a preference for hydrophobic or basic amino acids.[4,5] Carboxypeptidase B2 (CPB2), also known as thrombin-activatable fibrinolysis inhibitor, is 1 of 2 carboxypeptidases in plasma. The inactive proenzyme (proCPB2) is synthesized by the liver (Figure 1A).[5] In plasma, proCPB2 is activated by plasmin bound to glycosaminoglycans or by the thrombin-thrombomodulin complex (Figure 1B).[6] Once activated, CPB2 cleaves C-terminal basic amino acids from various proteins/peptides. CPB2 can inhibit fibrinolysis by cleaving lysine residues from partially degraded fibrin, preventing the binding of fibrinolytic components (Figure 1C and D).[7-10] Additionally, CPB2 exhibits anti-inflammatory properties, as it cleaves the C-terminal

arginine residue of C3a and C5a, generating the metabolites $C3a_{desArg}$ and $C5a_{desArg}$, limiting their proinflammatory effects (Figure 1C and E).[5,11-15]

In healthy individuals, proCPB2 circulates in plasma at a wide concentration range.[16,17] Genetic factors, such as polymorphisms, account for a significant portion of the variation in plasma (pro)CPB2 levels.[18-20] CPB2 gene (*CPB2*) polymorphisms have not been studied in transplantation; however, given its anti-inflammatory as well as antifibrinolytic properties, altered circulating (pro)CPB2 levels could potentially affect graft vulnerability.[21-23] Moreover, considering the contribution of complement activation to allograft injury and dysfunction in kidney transplantation, CPB2 is of further interest because of its modulatory effect on this system.[1,24] Here, we used a cohort of renal transplant donors and recipients, using human genetics to examine the role of (pro)CPB2 in kidney transplantation (Figure 1F–I). Looking at functional *CPB2* polymorphisms in donor-recipient pairs and evaluating effect modification by polymorphisms in complement genes, we investigated the association between these polymorphisms and long-term graft survival, aiming to understand the impact of (pro)CPB2 on kidney transplant outcomes.

MATERIALS AND METHODS

Study Design and Subjects

Transplant recipients were enrolled who received a single kidney allograft between March 1993 and February 2008 at the University Medical Center Groningen, as described previously.[25-28] Exclusion criteria were lack of a DNA sample, retransplantation, technical complications during surgery, and loss of follow-up. A total of 1271 of 1430 donor-recipient kidney transplant pairs were included. All participants provided written informed consent. The Institutional Review Board approved the study protocol (METc 2014/077), which adhered to the Declaration of Helsinki. The endpoint of our study was graft loss during follow-up with a maximum of 15 y. Graft loss was defined as the need for dialysis or retransplantation.

DNA Extraction and Genotyping

Peripheral blood mononuclear cells were isolated from blood or splenocytes that were collected from both the donor and recipient. DNA was extracted with a Qiagen FlexiGene DNA AGF3000 Kit on an AutoGenFlex 3000 workstation (Autogen, Holliston, MA), as instructed by the manufacturer, and stored at –80 °C. Genotyping of the single-nucleotide polymorphisms (SNPs) was determined via the Illumina VeraCode GoldenGate Assay kit (Illumina, San Diego, CA), according to the manufacturer's instructions. The promoter of *CPB2* contains several polymorphisms, of which the rs2146881 (–438 G>A) *CPB2* SNP is the most extensively studied. The A-allele of this polymorphism has been associated with lower plasma levels and activity of (pro)CPB2.[18,29-39] In addition, we chose the rs3742264 (505 G>A, Ala147Thr) and the rs1926447 (1040 C>T, Thr325Ile) *CPB2* SNPs. The Thr147 variant of the rs3742264 polymorphism ($CPB2_{147T}$ variant) has been associated with higher plasma levels and activity of (pro)CPB2,[18,29,32-35,37,38,40-46] whereas the Ile325 variant of the rs1926447 is linked to lower (pro)CPB2 levels.[18,29-35,37,43,47-51] However, the Ile325 variant also exhibits a 2-fold longer half-life and demonstrates greater effectiveness in inhibiting fibrinolysis compared with the Thr325 variant.[52] Finally, we included the rs3818477 (A>C, i4 + 164) *CPB2* SNP, of which the C-allele has previously been shown to associate with lower proCPB2 activity levels.[29] Altogether, the relationship between these CPB2 SNPs and their plasma levels has been studied extensively in different diseases (**Table S1**). Additionally, we genotyped 2 common functional polymorphism in the C3 and C5 genes: (1) the rs2230199 C>G (Gly102Arg) *C3* SNP and (2) the rs17611 G>A (2404 G/A, Val802Ile) *C5* SNP.[53,54] We assessed the combinations of the A-allele of the *CPB2* rs3742264 (referred to as the $CPB2_{147T}$ variant) with the GG-genotype of the *C3* SNP (referred to as $C3_{102G}$ variant) or the AA-genotype of the *C5* SNP (referred to as $C5_{V802}$ variant). Genotype clustering and calling were performed using BeadStudio Software (Illumina).

Genetic Risk Score

We compiled a polygenic CPB2 risk score for graft loss based on the 4 *CPB2* polymorphisms in the donor and the recipient. For each polymorphism, we categorized the minor allele as either protective or hazardous based on its association with graft loss in our cohort, and we determined this separately for the minor allele in the donor and recipient. We determined this separately for the presence of the donor and recipient. To account for the varying degrees of association between the polymorphisms and graft loss, we created a weighted risk score.[55] This score involves multiplying the point assigned for the presence of a minor allele by its corresponding regression coefficient, representing the logarithm of the hazard ratio (HR). A larger regression coefficient indicates a stronger effect of the minor allele on allograft survival. When a minor allele is protective, its regression coefficient is negative, and when it is hazardous, it is positive. The final genetic risk score is the sum of these weighted values for all 4 CPB2 polymorphisms in both the donor and recipient. In summary, a CPB2 risk score >0 indicates the presence of more hazardous alleles in a donor-recipient pair, whereas a score <0 indicates the presence of more protective alleles in such a pair.

Statistical Analysis

Data were presented as mean ± SD, median (interquartile range), or total number with percentage. Two-group comparisons used the Mann-Whitney *U* test, Student *t* test, or χ^2 test. CPB2 genotypes were analyzed for their association with 15-y death-censored graft survival using Kaplan-Meier analysis and log-rank testing. Cox proportional hazards regression analysis with stepwise adjustments for relevant clinical variables examined CPB2 genotypes' relationship with graft loss. CPB2 genotypes' association with graft loss was also assessed in subgroups using Cox proportional hazards regression analysis. Subgroups for continuous variables were based on values below versus above the median or mean.

Harrell's concordance statistic (c-statistic) was determined to evaluate the model's ability to differentiate between transplant recipients who experienced graft loss and those who did not, considering follow-up duration.[56] As our outcome variable graft loss is dichotomous, Harrell's c-statistic corresponds to the area under the receiver operating characteristic curve.[57] The improvement in predictive models for graft loss by the CPB2 genetic risk score was assessed using the integrated discrimination improvement (IDI). The IDI measures the new

TABLE 1.

Baseline characteristics of the donors and recipients

	All patients (N = 1271)	Functioning graft (N = 1056)	Graft loss (N = 215)	*P*[a]	HR	*P*[b]
Recipient						
CPB2 rs2146881 Polymorphism, n (%)						
GG	1221 (96.2)	1017 (96.5)	204 (94.9)	0.26		0.17
GA	48 (3.8)	37 (3.5)	11 (5.1)			
AA	0 (0)	0 (0)	0 (0)			
CPB2 rs3742264 polymorphism						
GG	571 (44.9)	465 (44.0)	106 (49.3)	0.36		0.26
GA	569 (44.8)	481 (45.5)	88 (40.9)			
AA	131 (10.3)	110 (10.4)	21 (9.8)			
CPB2 rs1926447 polymorphism						
CC	618 (48.8)	513 (48.8)	105 (48.8)	0.06		0.68
TC	547 (43.2)	455 (43.3)	92 (42.8)			
TT	101 (8.0)	83 (7.9)	18 (8.4)			
CPB2 rs3818477 polymorphism						
AA	493 (38.8)	405 (38.4)	88 (40.9)	0.66		0.45
AC	608 (47.8)	507 (48.0)	101 (47.0)			
CC	170 (13.4)	144 (13.6)	26 (12.1)			
Female sex, n (%)	532 (41.9)	449 (42.5)	83 (38.6)	0.29		0.21
Age, y	47.9 ± 13.5	48.5 ± 13.4	45.0 ± 13.2	**<0.001**	0.99	**0.027**
Dialysis vintage, wk	172 (91–263)	174 (87–261)	168 (109–270)	0.15		0.10
Blood group donor, n (%)						
Type O	567 (44.6)	474 (44.9)	93 (43.3)	**0.004**	0.46	**0.002**
Type A	536 (42.2)	448 (42.4)	88 (40.9)		0.46	**0.002**
Type B	113 (8.9)	98 (9.3)	15 (7.0)		0.35	**0.002**
Type AB	55 (4.3)	36 (3.4)	19 (8.8)		Ref	**0.008**
Immunosuppression, n (%)						
Anti-CD3 Moab	19 (1.5)	14 (1.3)	5 (2.3)	0.27		0.51
ATG	103 (8.1)	79 (7.5)	24 (11.2)	0.07		0.14
Azathioprine	72 (5.7)	53 (5.0)	19 (8.8)	**0.027**		0.29
Corticosteroids	1201 (94.5)	1002 (94.9)	199 (92.6)	0.17	0.51	**0.01**
Cyclosporine	1085 (85.4)	911 (86.3)	174 (80.9)	**0.044**	0.66	**0.016**
Interleukin-2 RA	199 (15.7)	163 (15.4)	36 (16.7)	0.63		0.12
Mycophenolic acid	907 (71.4)	775 (73.4)	132 (61.4)	**<0.001**		0.06
Sirolimus	38 (3.0)	33 (3.1)	5 (2.3)	0.53		0.54
Tacrolimus	97 (7.6)	77 (7.3)	20 (9.3)	0.31		0.39
Donor						
CPB2 rs2146881 polymorphism, n (%)						
GG	680 (54.8)	556 (54.0)	124 (58.5)	0.47		0.19
GA	478 (38.5)	402 (39.1)	76 (35.8)			
AA	83 (6.7)	71 (6.9)	12 (5.7)			
CPB2 rs3742264 polymorphism, n (%)						
GG	577 (45.5)	462 (43.8)	115 (53.5)	**0.033**	0.78	**0.022**
GA	574 (45.2)	490 (46.5)	84 (39.1)			
AA	118 (9.3)	102 (9.7)	16 (7.4)			
CPB2 rs1926447 polymorphism, n (%)						
CC	617 (48.6)	516 (48.9)	101 (47.2)	0.38		0.92
TC	538 (42.4)	440 (41.7)	98 (45.8)			
TT	114 (9.0)	99 (9.4)	15 (7.0)			
CPB2 rs3818477 polymorphism, n (%)						
AA	459 (36.2)	389 (36.9)	70 (32.6)	0.30		0.16
AC	637 (50.2)	527 (50.0)	110 (51.2)			
CC	172 (13.6)	137 (13.0)	35 (16.3)			
Female sex, n (%)	626 (49.3)	521 (49.3)	105 (48.8)	0.89		0.96
Age, y	44.4 ± 14.4	44.1 ± 14.6	46.1 ± 13.4	**0.044**	1.02	**<0.001**
Donor type, n (%)						
Living	282 (22.2)	257 (24.3)	25 (11.6)	**<0.001**	Ref	**0.002**
Deceased	989 (77.8)	642 (75.7)	190 (88.4)		1.94	

(*Continued*)

TABLE 1.

Continued

	All patients (N = 1271)	Functioning graft (N = 1056)	Graft loss (N = 215)	P^a	HR	P^b
Blood group, n (%)						
Type O	642 (50.6)	541 (51.3)	101 (47.2)	**0.033**	0.39	**0.004**
Type A	502 (39.6)	414 (39.3)	88 (41.1)		0.42	**0.01**
Type B	97 (7.6)	82 (7.8)	15 (7.0)		0.36	**0.012**
Type AB	27 (2.1)	17 (1.6)	10 (4.7)		Ref	**0.035**
Transplantation						
Highest PRA, %	10.1 ± 23.6	9.98 ± 23.7	10.9 ± 25.0	0.54		0.75
Total HLA mismatches	2 (1–3)	2 (1–3)	2 (1–3)	0.48		0.11
CIT, h	17.7 (10.9–23.0)	17.0 (8.6–23.0)	20.0 (15.3–25.0)	**<0.001**	1.03	**0.001**
WIT, min	37.0 (31–45)	37.0 (30–45)	38.0 (32–45)	0.12	1.02	**0.003**
DGF, n (%)	415 (32.7)	289 (27.4)	126 (58.6)	**<0.001**	3.79	**<0.001**

Bold values indicate *P* values that are statistically significant ($P < 0.05$).

The baseline demographics of all donor-recipient transplant pairs as well as subgroup analysis for graft loss after 15 y of follow-up. Data are displayed as mean ± SD, median (IQR), and the total number of patients with percentage.

[a]The *P* value for the differences in baseline demographics among the groups, tested by the Mann-Whitney *U* test or Student *t* test for continuous variables and χ^2 test for categorical variables.

[b]The *P* value for univariable analysis for graft loss after 15 y of follow-up.

ATG, antithymocyte globulin; CD3, cluster of differentiation 3; CIT, cold ischemia time; CPB2, carboxypeptidase B2; DGF, delayed graft function; IQR, interquartile range; PRA, panel-reactive antibody; RA, receptor antagonist; WIT, warm ischemia time.

TABLE 2.

Genotypic frequencies of *CPB2* polymorphisms in donors and recipients

CPB2	Donor	Recipient	P^a	1000 genome project	P^b	P^c
rs2146881						
GG	54.8 (680)	96.2 (1221)	**<0.0001**	57.1 (287)	0.62	**<0.0001**
GA	38.5 (478)	3.8 (48)		37.2 (187)		
AA	6.7 (83)	0 (0)		5.8 (29)		
rs3742264						
GG	45.5 (577)	44.9 (571)	0.69	44.3 (223)	0.37	0.75
GA	45.2 (574)	44.8 (569)		44.1 (222)		
AA	9.3 (118)	10.3 (131)		11.5 (58)		
rs1926447						
CC	48.6 (617)	48.8 (618)	0.65	48.1 (242)	0.60	0.22
CT	42.4 (538)	43.2 (547)		41.4 (208)		
TT	9.0 (114)	8.0 (101)		10.5 (53)		
rs3818477						
AA	36.2 (459)	38.8 (493)	0.39	41.0 (206)	**0.02**	0.10
AC	50.2 (637)	47.8 (608)		42.7 (215)		
CC	13.6 (172)	13.4 (170)		16.3 (82)		

A total of 1271 donor-recipient renal transplant pairs were analyzed for the presence of genetic variants in the *CPB2* gene. The frequencies of these polymorphisms were compared with those reported by the European cohort of the 1000 genomes project. Genotype frequencies are displayed as percentages with the corresponding total number of patients (% [n]). Bold *P*-values indicate *P*-values that are statistically significant (*P*-value < 0.05).

[a]*P* value for the Pearson chi-square test for differences in the genotype frequency between donors and recipients.

[b]*P* value for the Pearson chi-square test for differences in the genotype frequency between donors and the European cohort of the 1000 genome project.

[c]*P* value for the Pearson chi-square test for differences in the genotype frequency between recipients and the European cohort of the 1000 genome project.

CPB2, carboxypeptidase B2.

model's ability to enhance average sensitivity without reducing average specificity.[56,58] Statistical testing was 2-tailed, and significance was defined as *P* value of <0.05. Statistical analyses were conducted using SPSS software version 25 (SPSS Inc, Chicago, IL) and STATA Statistical Software: Release 17 (StataCorp., College Station, TX).

RESULTS

Patient Population

During a mean follow-up period of 6.2 ± 4.2 y, 215 kidney transplant recipients lost their grafts (16.9%), whereas 191 recipients died with a functioning graft (15.0%). Deceased donor kidney transplantation, absence of cyclosporine and corticosteroids for immunosuppression, and donor and recipient blood type AB were significantly more prevalent in patients who progressed to graft loss. Furthermore, patients with graft loss were younger, whereas their donors were older, and both cold and warm ischemia times were significantly longer. Finally, delayed graft function occurred more frequently in patients developing graft loss (Table 1).

Among the *CPB2* polymorphisms, no differences were seen in genotypic frequencies between the donors, recipients, and the European cohort of 1000 genomes project for rs3742264 and rs1926447 (Table 2). However, the minor allele frequency of the rs2146881 SNP was significantly

lower in recipients compared with donors ($P < 0.0001$) and the European cohort of the 1000 genomes project ($P < 0.0001$, Table 2). This discrepancy raises the potential of a survival bias. Furthermore, the distribution of the rs2146881 polymorphism in recipients deviated from Hardy-Weinberg equilibrium, suggesting an association of this SNP with kidney failure.

The *CPB2*$_{147T}$ Variant Associates With Graft Loss After Kidney Transplantation

We first examined whether polymorphisms in *CPB2* were associated with graft loss after kidney transplantation and found that the rs3742264 polymorphism in the donor was significantly associated with 15-y death-censored graft survival ($P = 0.022$). Furthermore, all-cause mortality was similar among the CPB2 genotypes, confirming that patient survival was not a confounder in the association with graft loss (Table 3). Kaplan-Meier survival analysis revealed that the *CPB2*$_{147T}$ variant of the rs3742264 SNP in the donor was associated with a lower risk of graft loss after transplantation (Figure 2A; $P = 0.044$). The cumulative incidence of graft loss after 15 y of follow-up was 19.9% in the reference *CPB2*$_{147A/A}$ group, 14.6% in the *CPB2*$_{147A/T}$ group, and 13.3% in the *CPB2*$_{147T/T}$ group, respectively. For further analysis, the *CPB2*$_{147T/T}$ and *CPB2*$_{147T/T}$ were combined into 1 group (Figure 2B, $P = 0.013$) because the incidence of graft loss was not significantly different between these genotypes. In univariable analysis, carrying at least 1 *CPB2*$_{147T}$ variant in the donor was significantly associated with improved 15-y death-censored graft survival (HR, 0.71; 95% confidence interval [CI], 0.55-0.93; $P = 0.014$). Multivariable models were constructed using a stepwise forward selection procedure, including all variables that were significantly associated with graft loss in the univariable analysis (Table 4). In the final model, the *CPB2* rs3742264 SNP in the donor, recipient age, the occurrence of delayed graft function, recipient blood type, and donor age were included. After adjustment, the *CPB2*$_{147T}$ variant in the donor was significantly associated with a reduced risk of graft loss (HR, 0.67; 95% CI, 0.51-0.88; $P = 0.004$).

Furthermore, we performed a subgroup analysis for the donor type because deceased organ donors are often characterized by coagulopathies.[59] Kaplan-Meier curves demonstrated that the association remained significant between the *CPB2* rs3742264 SNP in the donor and long-term graft survival for kidney allografts from deceased donors (Figure 2C; $P = 0.012$). However, the association lost statistical significance for kidney allografts from living donors (Figure 2D; $P = 0.84$). Our results show that the *CPB2*$_{147T}$ variant in deceased organ donors is associated with a reduced risk of graft loss after kidney transplantation.

TABLE 3.

Patient mortality according to *CPB2* polymorphism genotypes

CPB2	Donor	*P*[a]	Recipient	*P*[b]
rs2146881				
GG	17.1 (116)	0.18	17.0 (208)	0.29
GA	19.0 (91)		22.9 (11)	
AA	10.8 (9)		0 (0)	
rs3742264				
GG	94 (16.3)	0.43	16.8 (96)	0.87
GA	18.8 (108)		17.9 (102)	
AA	15.3 (18)		16.8 (22)	
rs1926447				
CC	17.7 (95)	0.62	16.8 (104)	0.20
CT	17.7 (95)		16.6 (91)	
TT	14.0 (16)		23.8 (24)	
rs3818477				
AA	17.7 (95)	0.35	18.1 (89)	0.52
AC	17.7 (95)		16.1 (98)	
CC	14.0 (16)		19.4 (33)	

A total of 1271 donor-recipient renal transplant pairs were analyzed for the presence of genetic variants in the *CPB2* gene. All-cause mortality was compared among the genotypes of these polymorphisms. Morality rates are displayed as percentages with the corresponding total number of patients (% [n]).

[a]*P* value for the Pearson chi-square test for differences in all-cause mortality among the donor genotypes.

[b]*P* value for the Pearson chi-square test for differences in all-cause mortality among the recipient genotypes.

CPB2, carboxypeptidase B2.

CPB2 Associates With Graft Loss Potentially Through Inactivation of Anaphylatoxins

Next, we investigated if the association between the *CPB2*$_{147T}$ variant and long-term graft survival was mediated via ability of CPB2 to inactivate complement anaphylatoxin C3a and C5a. On this basis, we tested the combined effect of the *CPB2*$_{147T}$ variant with gain-of-function complement polymorphisms on graft loss after kidney transplantation.

First, we simultaneously analyzed the *CPB2*$_{147T}$ variant with a common functional polymorphism in the C3 gene (*C3*) rs2230199, resulting in a glycine to arginine substitution at position 102. The minor allele of this polymorphism results in a *C3* variant ($C3_{102G}$) that is less well inhibited by Factor H, a complement regulator, leading to increased C3 activation and higher C3a formation.[60] Next, donor-recipient pairs were separated into 4 groups according to the presence or absence of the *C3* and *CPB2* polymorphism in the donor. Kaplan-Meier survival analyses showed a significant difference in graft failure rates among the 4 groups ($P = 0.022$; Figure 3A). Kidney allografts possessing the *CPB2*$_{147T}$ variant and the reference $C3_{102R}$ variant had the best outcome (15-y death-censored graft survival: 77.2%), whereas kidney allografts possessing the $C3_{102G}$ variant and the reference *CPB2*$_{147A}$ variant had the worst outcome (15-y death-censored graft survival: 50.2%). Moreover, kidney allografts possessing both variants and no variants showed similar outcomes, suggesting that the protective effect of the *CPB2*$_{147T}$ variant could be mitigated by the hazardous effect of the $C3_{102R}$ variant.

Next, we simultaneously analyzed the *CPB2*$_{147T}$ variant with a common functional polymorphism in the C5 gene (*C5*) rs17611, resulting in a valine to isoleucine substitution at position 802. The G-allele of this polymorphism results in a *C5* variant ($C5_{V802}$) that is more susceptible to cleavage by proteases, leading to enhanced C5a production.[61] Once again, donor-recipient pairs were separated into 4 groups according to the presence or absence of the *C5* and *CPB2* polymorphism in the donor. Kaplan-Meier survival analyses showed a significant difference in graft failure rates among the 4 groups ($P = 0.009$; Figure 3B). Kidney allografts possessing the *CPB2*$_{147T}$ variant and the reference $C5_{I802}$ variant had the best outcome (15-y death-censored graft survival: 78.7%), whereas kidney allografts possessing the $C5_{V802}$ variant and

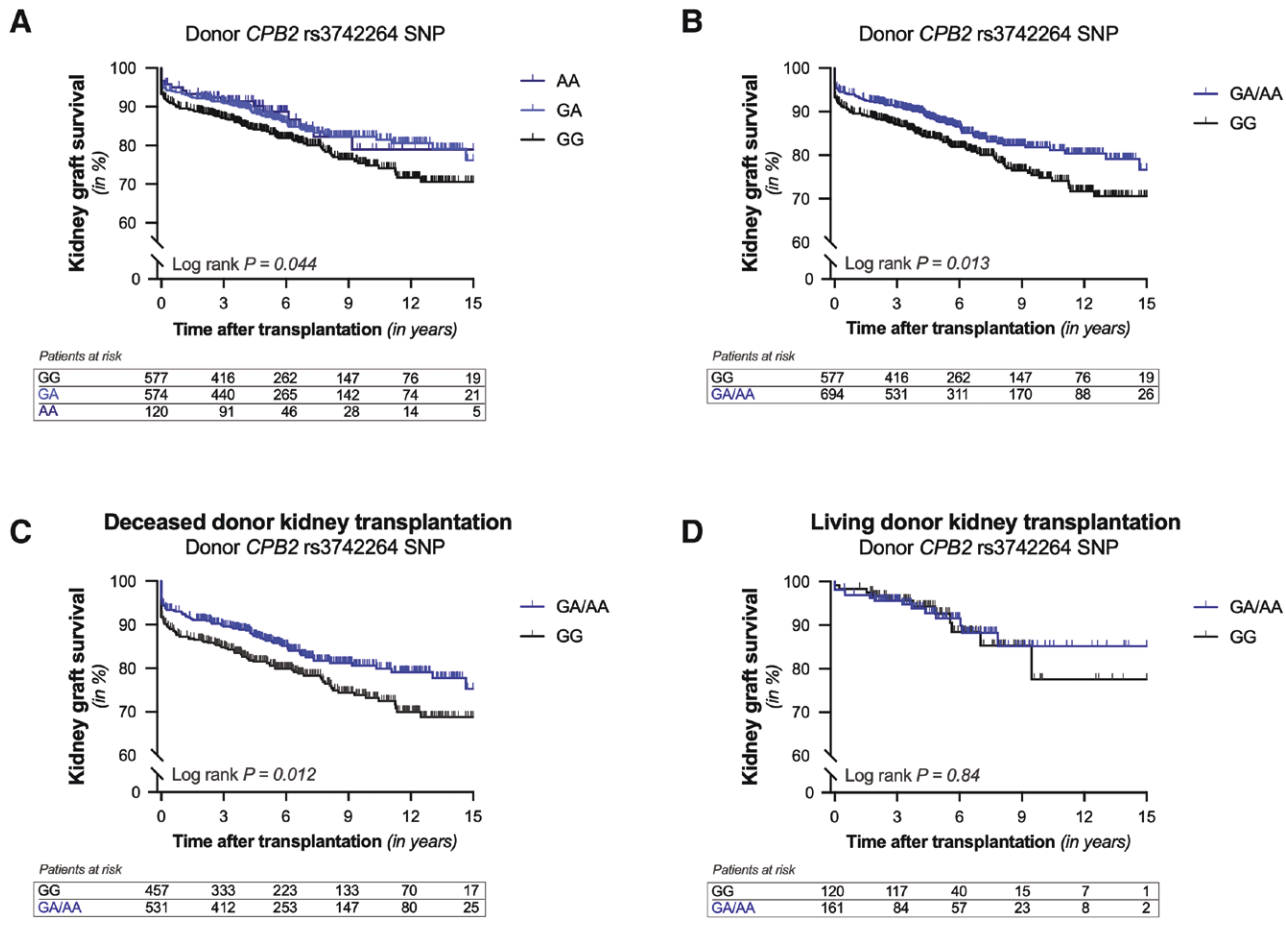


FIGURE 2. Kaplan-Meier curves for 15-y death-censored kidney graft survival according to the presence of a *CBP2* gene polymorphism in the donor. A, Cumulative 15-y death-censored kidney graft survival with the rs3742264 G>A ($CPB2_{1A147T}$) polymorphism in the *CBP2* gene in the donor. B, Next, the GA- and AA-genotype were combined in 1 group because the graft loss incidence did not significantly differ between these genotypes. A subgroup analysis for donor type was performed. Cumulative 15-y death-censored kidney graft survival with the rs3742264 G>A polymorphism in deceased kidney donors (C) and living kidney donors (D). The log-rank test was used to compare the graft loss incidence between the different groups. CBP2, carboxypeptidase B2; SNP, single-nucleotide polymorphism.

the reference $CPB2_{147A}$ variant had the worst outcome (15-y death-censored graft survival: 53.2%). Moreover, kidney allografts possessing both variants and no variants experienced similar outcomes, suggesting once again the protective effect of the $CPB2_{147T}$ variant could be mitigated by the hazardous effect of the $C5_{V802}$ variant. In conclusion, our data imply that the association of the $CPB2_{147T}$ variant with graft loss is possibly mediated via ability of CPB2 to inactivate complement anaphylatoxin C3a and C5a.

A *CPB2* Genetic Risk Score Improves Risk Prediction for Graft Loss

In addition to analyzing the *CPB2* polymorphisms separately, we computed a genetic risk score for each donor-recipient for all *CPB2* variants, assigning weights to the minor alleles based on their HRs, with positive coefficients for hazardous alleles and negative coefficients for protective alleles. The CPB2 risk score is the sum of these weighted values for all 4 CPB2 polymorphisms in both the donor and recipient. The CPB2 genetic risk score was significantly associated with 15-y death-censored graft survival in univariable analysis (HR, 1.30; 95%-CI, 1.14-1.49; $P < 0.001$ per SD increase). Multivariable analysis was performed with stepwise adjustments for additional relevant clinical variables (Table 5), including recipient characteristics (model 2), donor characteristics (model 3), and transplant variables (model 4). In Cox regression analysis, the CPB2 genetic risk score remained significantly associated with graft loss independent of potential confounders. Thereafter, we investigated whether the CPB2 genetic risk score (including the *CPB2* rs3742264 SNP) was a better predictor of graft loss than the *CPB2* rs3742264 SNP in the donor alone by multivariable regression with a stepwise forward selection (Table 6). In the final model, the CPB2 genetic risk score containing the *CPB2* rs3742264 SNP was included, whereas the single *CPB2* rs3742264 SNP in the donor was excluded. After adjustment, the CPB2 genetic risk score was associated with graft loss with a HR of 1.31 per SD increase (95% CI, 1.14-1.50; $P < 0.001$). In addition, the HR of the CPB2 genetic risk score was consistent and remained significant in subgroup analyses (Figure 4), except for living donors and female recipients. The 95% CI of the various subgroups had substantially overlapped with the general HR, signifying the consistency of the impact of the CPB2 genetic risk score across the subgroups.

Finally, the performance of the CPB2 genetic risk score for the prediction of graft loss was also assessed (Table 7). The CPB2 genetic risk score had a Harrell's C of 0.59 (95% CI, 0.55-0.63). Moreover, when added to a model of the *CPB2*

TABLE 4.

Multivariable analysis of 15-y death-censored graft survival

Variables not in the equation		Variables in the equation		
Variables	***P***	**Variables**	***P***	**Hazard ratio**
Warm ischemia time, min	0.07	*CPB2*$_{147T}$ variant in the donor	0.004	0.67 (1.21-3.82)
Corticosteroids	0.09	Recipient age, y	<0.001	0.98 (0.97-0.99)
Cold ischemia time, h	0.11	Delayed graft function (yes vs no)	<0.001	4.02 (3.03-5.32)
Donor type (living vs deceased)	0.13	Recipient blood type (ABO vs other)	0.001	
Cyclosporine	0.35	Donor age, y	0.002	1.02 (1.01-1.03)
Donor blood type (ABO vs other)	0.98			

Multivariable Cox regression was performed with a stepwise forward selection. Only variables with a *P* value of <0.05 in the univariable analysis were included. Data are presented as hazard ratio with 95% CI and *P*-value. In the final model, the *CPB2* SNP (rs3742264-T) in the donor, recipient age, the occurrence of delayed graft function, recipient blood type, and donor age were included, whereas warm ischemia time, use of corticosteroids, cold ischemia time, donor type, use of cyclosporine, and donor blood type were not.
CI, confidence interval; CPB2, carboxypeptidase B2; SNP, single-nucleotide polymorphism.

s3742264 SNP in the donor (c-statistic, 0.55; 95% CI, 0.51-0.59), the CPB2 genetic risk score significantly improved the Harrell's C (c-statistic increase, 0.035; 95% CI, 0.005-0.066; $P = 0.02$). As additional variables were included and the discriminative accuracy to predict graft loss of the model improved. The Harrell's C of the models with the recipient, donor, and transplant characteristics significantly increased with the addition of the CPB2 genetic risk score (models 3–5), whereas only a trend was seen in the model with all variables that were significantly associated with graft loss in multivariable regression analysis (model 6). Finally, the CPB2 genetic risk score significantly increased the predictive value of the models according to the IDI.

DISCUSSION

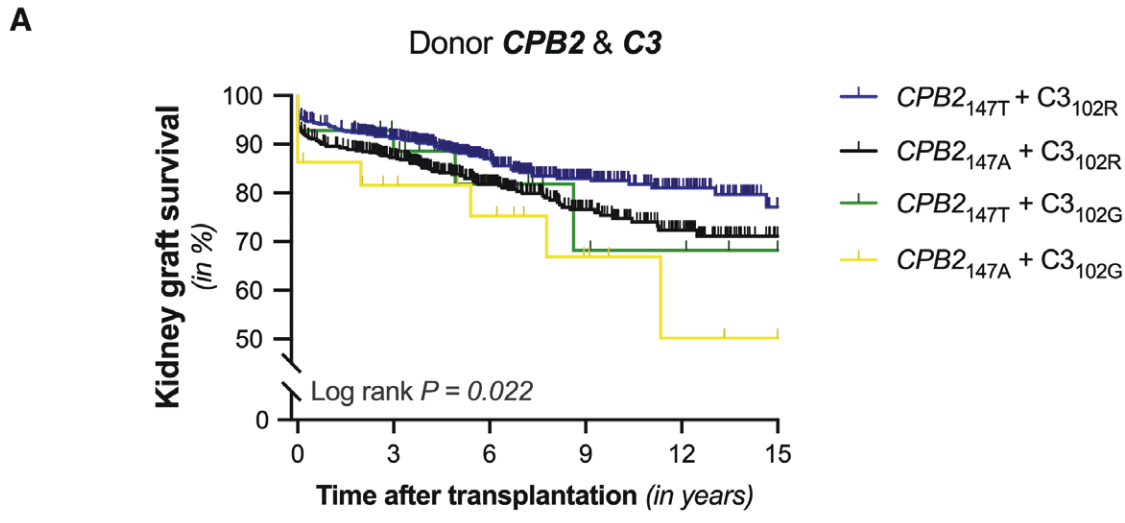
Here, we report that kidney transplantation from allografts possessing a *CPB2*$_{147T}$ variant results in a significantly attenuated risk of graft failure. We propose that the higher survival rate could be explained by the ability of CPB2 to inactive complement anaphylatoxins (ie, C3a and C5a) because the protective effect of the *CPB2* polymorphism on graft loss could be reversed by gain-of-function polymorphisms in *C3* and *C5*. Furthermore, we demonstrated that a genetic risk score based on CPB2 polymorphisms in donor-recipient pairs was an important determinant of long-term graft survival. Finally, our data suggest CPB2 as a novel treatment strategy to improve kidney transplantation outcomes, especially because therapeutic strategies informed by human genetic evidence have a significantly higher likelihood of leading to approved therapeutics.[62,63]

CPB2, because of its antifibrinolytic activity, has been studied in cardiovascular disease and thrombotic disorders.[17] Increased CPB2 levels are anticipated to induce a hypofibrinolytic state, representing a potential risk factor for various coagulation disorders. Accordingly, thrombus formation was ameliorated in mice deficient in *CPB2* (*Cpb2*$^{-/-}$) on ferric chloride ($FeCl_3$)-induced vena cava thrombosis.[64] Recently, a meta-analysis investigated the impact of *CPB2* variants on the risk of cardiovascular disease, failing to confirm a significant association.[65] Similarly, another meta-analysis examined the effect of *CPB2* polymorphisms on venous thrombosis risk found no significant association.[66]

Apart from having antifibrinolytic activity during clot formation, CPB2 also has an anti-inflammatory role.[5,15] These anti-inflammatory properties are mediated through the cleavage of C-terminal arginine from proinflammatory mediators, such as complement anaphylatoxins, bradykinin, osteopontin, and vascular endothelial growth factor-A.[5,11-14] Recent work demonstrates that CPB2 dampened inflammation in autoimmune arthritis.[67] After the induction of anticollagen antibody-induced arthritis, *Cpb2*$^{-/-}$ mice exhibited more severe arthritis, whereas *C5*$^{-/-}$ mice were protected.[67] Intriguingly, an anti-C5 antibody prevented the development of severe arthritis in *Cpb2*$^{-/-}$ mice. In accordance, *Cpb2*$^{-/-}$ mice also had a greater complement-mediated influx of inflammatory cells in a model of zymosan-induced peritonitis.[67] Furthermore, rheumatoid arthritis patients carrying a *CPB2* variant, resulting in a longer half-life of CPB2, were less likely to develop severe disease.[67] A similar relationship was found between CPB2 levels and complement activation in antiphospholipid syndrome.[68] Collectively, these studies demonstrate the profound ability of CPB2 to reduce inflammation, particularly through its ability to inactivate complement anaphylatoxins. As such, CPB2 belongs to a group of enzymes that bridge the coagulation system and the complement cascade, like C1 esterase inhibitor and thrombomodulin.[5,69-71]

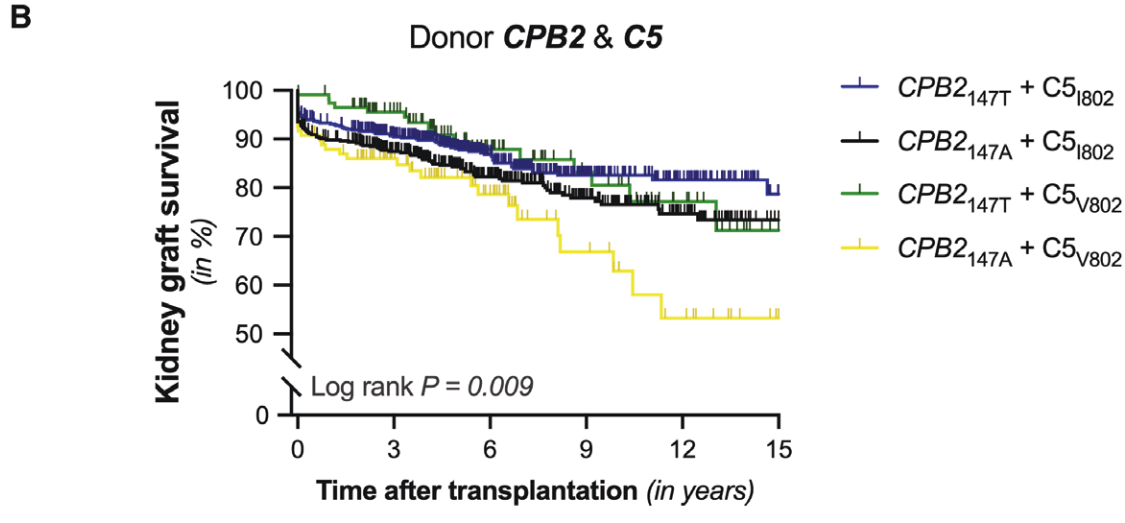
To our knowledge, our study is the first to explore the role of *CPB2* polymorphisms in kidney transplantation in relation to graft survival. Plasma (pro)CPB2 levels have been reported to be elevated in kidney transplant recipients compared with healthy controls.[72,73] Consequently, CPB2 has been postulated to contribute to the hypofibrinolysis seen in this patient population, thereby negatively impacting transplant outcomes. Microthrombi are frequently formed within the graft during kidney transplantation, which can then lead to poor initial kidney graft function.[59,74,75] However, we found that the rs3742264 *CPB2* polymorphism in kidney allografts reduced the risk of graft failure. This SNP has previously been associated with higher levels.[29,32-34,45] These findings were confirmed by a recent genome-wide linkage analysis performed in a European population, which revealed a significant correlation between the rs3742264 *CPB2* polymorphism and elevated plasma concentrations of both intact (pro)CPB2 and its activation peptide.[35] Hence, we hypothesized that the association found was mediated by ability of CPB2 to inactivate C3a and C5a, especially because complement anaphylatoxins are known to impact graft outcome in kidney transplantation.[1,53,76-79] To test this, we looked at the combined impact of the *CPB2*$_{147T}$ variant with *C3* or *C5* polymorphisms that have been reported to lead to higher C3a and C5a levels.[60,61] In line with our hypothesis, we found kidney allografts possessing the *CPB2*$_{147T}$ variant as well as a complement gain-of-function had almost identical outcomes compared with kidney allografts carrying neither variant. Therefore, we propose that in kidney transplantation, high (pro)CPB2 levels improve graft survival by reducing complement-mediated inflammation.

Carboxypeptidase N (CPN) and CPB2 are the only plasma carboxypeptidases.[5] The current paradigm is that CPN is the main regulator of C3a and C5a, whereas CPB2



Patients at risk

$CPB2_{147T}$	+ $C3_{102R}$	664	506	301	165	84	25
$CPB2_{147A}$	+ $C3_{102R}$	553	399	247	139	72	18
$CPB2_{147T}$	+ $C3_{102G}$	28	22	10	6	4	1
$CPB2_{147A}$	+ $C3_{102G}$	22	16	13	8	5	2



Patients at risk

$CPB2_{147T}$	+ $C5_{I802}$	568	434	256	137	70	23
$CPB2_{147A}$	+ $C5_{I802}$	464	341	215	126	65	18
$CPB2_{147T}$	+ $C5_{V802}$	116	91	53	32	18	3
$CPB2_{147A}$	+ $C5_{V802}$	108	71	43	21	11	1

FIGURE 3. Kaplan-Meier curves for 15-y death-censored kidney graft survival according to the presence of a *CBP2* and *C3* and *C5* gene polymorphism in the donor. A, Cumulative 15-y death-censored kidney graft survival according to the presence of the rs3742264 G>A polymorphism in the *CBP2* gene and the rs2230199 C>G polymorphism in the *C3* gene. Pairs were divided into 4 groups according to the absence (black line), presence of the CPB2 variant (blue line), presence of the C3 variant (yellow line), or both (green line). B, Cumulative 15-y death-censored kidney graft survival according to the presence of the rs3742264 G>A polymorphism in the *CBP2* gene and the rs17611 G>A polymorphism in the *C5* gene. Pairs were divided into 4 groups according to the absence (black line), presence of the CPB2 variant (blue line), presence of the C5 variant (yellow line), or both (green line). A log-rank test was used to compare the incidence of graft loss between the groups. CBP2, carboxypeptidase B2.

would be primarily involved in fibrinolysis. However, in vitro experiments revealed the ability of CPB2 to inactivate C3a and C5a.[13] Moreover, CPN is less efficient in cleaving C5a into $C5a_{DesArg}$ than CPB2, whereas for C3a, both enzymes were equally efficient.[13] Studies using *Cpb2*$^{-/-}$ mice confirmed that inactivation of anaphylatoxins is regulated by CPB2 in vivo. In a model of hemolytic uremic syndrome, the disease was exacerbated in *Cpb2*$^{-/-}$ mice, compared with controls and *Cpn*$^{-/-}$ mice.[11] Additionally, *Cpb2*$^{-/-}$ mice presented with the clinical hemolytic uremic syndrome triad

TABLE 5.

Associations of CPB2 genetic risk score with graft loss

	CPB2 genetic risk score		
	Hazard ratio (per SD)	95% CI	*P*
Model 1	1.302	1.140-1.488	<0.001
Model 2	1.309	1.139-1.505	<0.001
Model 3	1.279	1.118-1.463	<0.001
Model 4	1.268	1.102-1.458	0.001

Data are presented as the hazard ratio with a 95% CI and *P* value.
Model 1: crude model.
Model 2: adjusted for model 1 plus recipient characteristics: recipient age, recipient sex, recipient blood type, and dialysis vintage.
Model 3: adjusted for model 1 plus donor characteristics: donor age, donor sex, donor blood type, and donor origin.
Model 4: adjusted for model 1 plus transplant characteristics: cold and warm ischemia time, the total HLA mismatches, and the occurrence of DGF.
CI, confidence interval; CPB2, carboxypeptidase B2; DGF, delayed graft function.

TABLE 6.

Competitive analysis of clinical factor associations with graft loss

Variables not in the equation		Variables in the equation		
Variables	*P*	Variables	*P*	Hazard ratio
Cold ischemia time, h	0.10	*CPB2* genetic risk score (per SD)	<0.001	1.31 (1.14-1.50)
Warm ischemia time, min	0.14	Recipient age, y	<0.001	0.98 (0.97-0.99)
Donor type (living vs deceased)	0.17	Delayed graft function (yes vs no)	<0.001	3.80 (2.86-5.05)
$CPB2_{147T}$ variant in the donor	0.35	Recipient blood type (ABO vs other)	0.001	
Cyclosporine	0.83	Donor age, y	0.007	1.01 (1.00-1.02)
Donor blood type (ABO vs other)	0.99	Corticosteroids	0.035	0.56 (0.33-0.96)

Multivariable Cox regression was performed with a stepwise forward selection. Only variables with a *P* value of < 0.05 in the univariable analysis were included. Data are presented as hazard ratio with 95% CI and *P* value. In the final model, the *CPB2* genetic risk score, recipient age, occurrence of delayed graft function, recipient blood type, donor age, and use of corticosteroids were included, whereas cold ischemia time, warm ischemia time, donor type, *CPB2* SNP (rs3742264-T) in the donor, use of cyclosporine, and donor blood type were not.
CI, confidence interval; CPB2, carboxypeptidase B2; SNP, single-nucleotide polymorphism.

of thrombocytopenia, uremia, and microangiopathic hemolytic anemia.[11] The exacerbated disease in *Cpb2*$^{-/-}$ mice was attenuated by the treatment with an anti-C5 antibody, and survival after treatment was comparable with that of wild-type mice.[11] Moreover, patients with a history of thrombotic microangiopathy were found to have a significantly higher prevalence of *CPB2* polymorphism alleles associated with lower levels.[80] Altogether, our study adds to a growing body of evidence that implicates CPB2 as a chief regulator of C3a and C5a.

Regarding donor-recipient nuances, we found an association between a *CPB2* polymorphism in donors with the risk of graft loss but not recipients. These results reveal that it is not (pro)CPB2 production by the recipient but instead the local (pro)CPB2 production by the donor kidney that positively impacts graft survival in kidney transplantation. Furthermore, when we performed a subgroup analysis for donor type, we observed a significant association between the $CPB2_{147T}$ variant in deceased donors and graft loss. Although a similar protective effect was seen for the $CPB2_{147T}$ variant in living donors, this was not significant. Donor kidneys from deceased organ donors have inferior outcomes and lower graft survival rates than kidneys retrieved from living donors.[81] These discrepancies are attributed to the immunological activation, systemic inflammation, and coagulopathies seen in deceased organ donors.[1,24,59] It is therefore not surprising that the protective effect of the $CPB2_{147T}$ variant may be stronger in deceased organ donors, especially because C5a levels are higher in these donors compared with their living counterparts.[76,82] Our findings also suggest that donor pretreatment with (pro)CPB2 might be a promising strategy to increase graft function and survival. Recently, a recombinant homolog of CPB2 was shown to alleviate vascular cell damage by decreasing C3a- and C5a-induced neutrophil extracellular trap formation and suggested as an early intervention for COVID-19.[83] Alternatively, rather than enhancing (pro)CPB2 levels, blocking the generation of C3a and C5a through a complement inhibitor could be a better approach.[77-79]

The strengths of this study include the exploration of common functional *CPB2* variants in a large sample size of donor-recipient pairs, the joint analysis of *CPB2* and complement polymorphisms, the clinically meaningful endpoint, and the comprehensive and extended follow-up period. Nonetheless, there are certain limitations to consider. Our study, being observational, cannot definitively prove causal relationships despite the expected associations. Furthermore, we cannot exclude that the *CPB2* rs3742264 polymorphism might be a tag SNP for other *CPB2* variants due to high linkage disequilibrium.[35] Third, the unavoidable index event bias inherent to the study design—arising from analyzing risk factors (CPB2 polymorphisms) in a population enriched for a specific disease (kidney failure) and its potential impact on associations with disease-related outcomes (graft loss)—could distort true associations of recipient *CPB2* genotypes with graft loss, resulting in false-negative findings. The fact that this study is conducted at a single center limits the generalizability of our findings to other centers and regions. Fifth, we did not account for the increase in familywise error rate across the reported statistical analyses. Sixth, we were unable to study the relationship between the polymorphisms and (pro)CPB2 levels in the circulation or kidneys due to the absence of plasma and biopsy samples. Nevertheless, it is worth noting that the $CPB2_{147T}$ variant, associated with a lower risk of graft loss in our study, has consistently been linked to higher (pro)CPB2 plasma levels in numerous studies across various diseases.[18,29,32-35,37,38,40-46] Additionally, previous research has demonstrated (pro)CPB2 both at protein level and gene expression in rodent and human kidneys.[84-90] More specifically, using the Gene Expression Omnibus database from Fletcher et al, mRNA expression of proCPB2 is seen in biopsies from normal donor kidneys, well-functioning transplants without rejection, kidneys undergoing acute rejection, and transplants with renal dysfunction without rejection.[90] Furthermore, increased mRNA proCPB2 expression has been observed in kidneys during pathological conditions compared with healthy ones.[84] Moreover, proteomic studies have specifically identified (pro)CPB2 in deceased human donor kidneys, including in tissue, urine, and perfusate.[84-87] Therefore, while the liver is the primary source of proCPB2, the kidney also seems capable of producing it. Similarly, proCPB2 can be synthesized by macrophages and platelets.[67,91] Finally, for >2 decades, there has

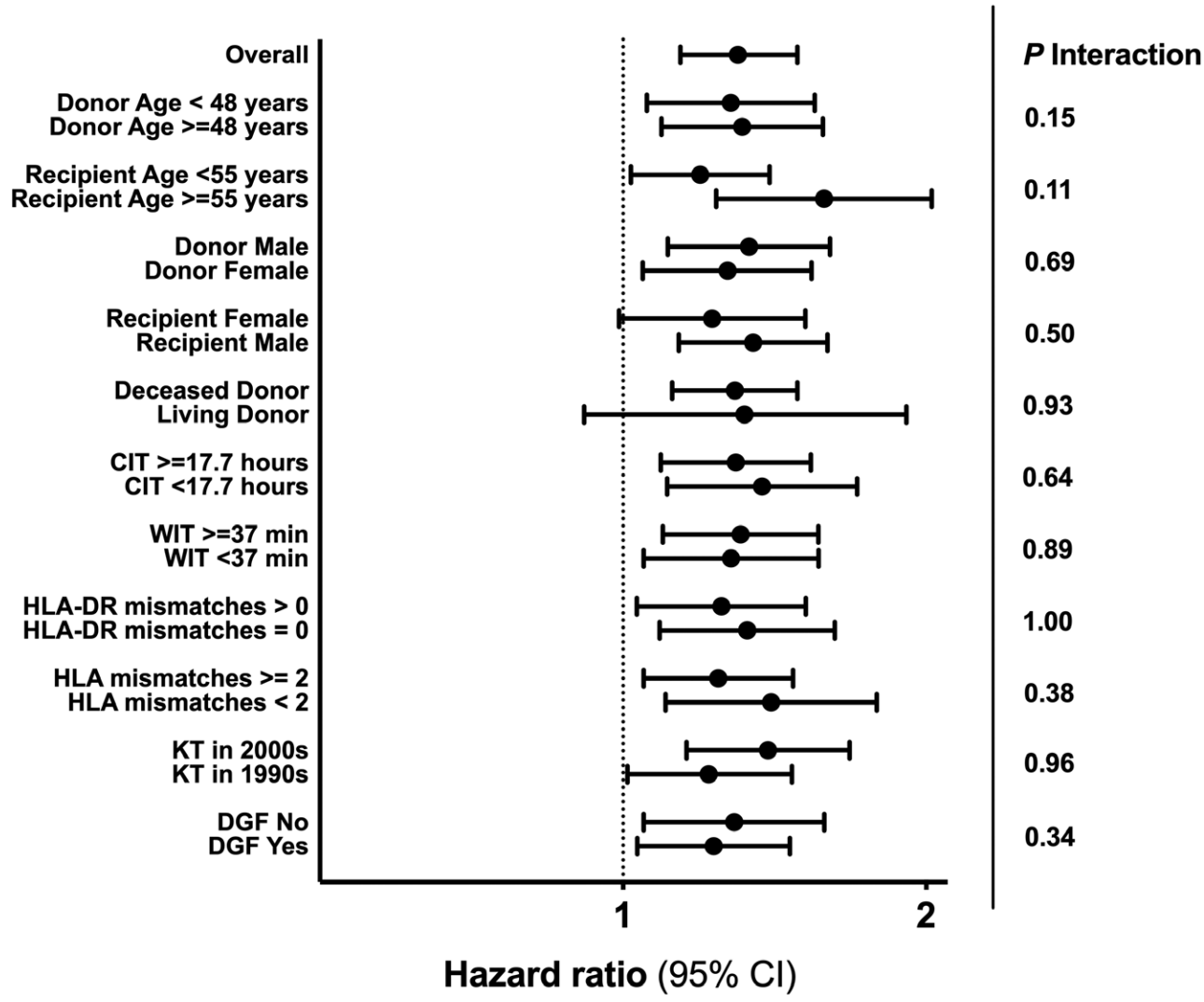


FIGURE 4. Hazard ratios for CPB2 genetic risk score among subgroups. Forest plot of CPB2 genetic risk score subanalyses, demonstrating the consistency of the hazard ratios for graft loss in the different subgroups with the exception of donor origins of the kidney allografts and recipient sex. The association between the CPB2 genetic risk score and graft loss was not observed in KTs from living donors or female recipients. No significant interaction was seen between the CPB2 genetic risk score and the various clinical variables of the subgroups. CBP2, carboxypeptidase B2; CIT, cold ischemia time; DGF, delayed graft function; KT, kidney transplant; WIT, warm ischemia time.

TABLE 7.

Additive value of the *CPB2* genetic risk score for the prediction of graft loss

	Harrell's C (95% CI)					
	Without the CPB2 genetic risk score	With the CPB2 genetic risk score	Change[a] (95% CI)	*P*	IDI, %	*P*
Model 1	0.500	0.588 (0.546-0.629)	NA	NA	NA	NA
Model 2	0.550 (0.514-0.586)	0.585 (0.544-0.626)	0.035 (0.005-0.066)	0.02	0.7	0.003
Model 3	0.566 (0.525-0.607)	0.615 (0.575-0.656)	0.051 (0.016-0.086)	0.004	1.2	0.001
Model 4	0.623 (0.587-0.660)	0.645 (0.608-0.682)	0.028 (0.003-0.053)	0.03	1.0	0.003
Model 5	0.704 (0.668-0.740)	0.722 (0.685-0.759)	0.021 (0.000-0.042)	0.06	1.0	0.005
Model 6	0.734 (0.700-0.769)	0.744 (0.711-0.777)	0.012 (-0.001-0.025)	0.08	1.1	0.007

Data are presented as Harrell's concordance statistic with a 95% CI and IDI with a *P* value.
Model 1: crude model.
Model 2: CPB2 rs3742264 polymorphism in the donor.
Model 3: adjusted for recipient characteristics: recipient age, recipient sex, recipient blood type, and dialysis vintage.
Model 4: adjusted for donor characteristics: donor age, donor sex, donor blood type, and donor origin.
Model 5: adjusted for plus transplant characteristics: cold and warm ischemia time, the total HLA mismatches, and the occurrence of delayed graft function.
Model 6: adjusted for the occurrence of delayed graft function, recipient blood type, donor age, and use of corticosteroids.
[a]Change in c-statistics compared with the model without the CPB2.
CI, confidence interval; CPB2, carboxypeptidase B2; IDI, integrated discrimination improvement.

been a growing body of evidence demonstrating the intrarenal synthesis of complement by various cell types within the kidney.[92] More specifically, in its normal state, donor kidneys have been shown to generate approximately 5% of the circulating C3, which was shown to increase to 16% during rejection.[93] Future analyses should further explore the contribution of the (donor) kidney to circulating (pro)CPB2 as well as the main cell type(s) responsible for the renal synthesis of (pro)CPB2 and C3.

In conclusion, we found that patients receiving a donor kidney carrying the $CPB2_{147T}$ variant have a lower risk of late graft loss. Considering the $CPB2_{147T}$ variant has been associated with higher protein levels, our findings imply a potential beneficial effect of CPB2 on long-term allograft survival in renal transplantation. We propose that the association between the donor genotype and graft loss stems from the specific impact of local (pro)CPB2 levels within the kidney. Additionally, we hypothesize that under normal conditions, (pro)CPB2 levels in the kidney are low, whereas the $CPB2_{147T}$ variant results in significant expression in the donor kidney. This would explain why CPB2 polymorphisms associated with lower levels, or the recipient genotype, do not affect graft loss risk. Future studies should explore this hypothesis. Overall, we consider this study preliminary and encourage replication.

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