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ORIGINAL ARTICLE

ANRIL as a genetic marker for cardiovascular events in renal transplant patients – an observational follow-up cohort study

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SUMMARY

Cardiovascular disease is the leading cause of morbidity and mortality in kidney transplant recipients. Several single-nucleotide polymorphisms (SNPs) in the *ANRIL* gene pathway have been associated with cardiovascular events (CE). The main objective was to ascertain whether *ANRIL* (rs10757278) and *CARD8* (rs2043211) SNPs could mediate susceptibility to CE. This was an observational follow-up cohort study of renal transplant recipients at Bellvitge University Hospital (Barcelona) from 2000 to 2014. A total of 505 recipients were followed up until achievement of a CE. Patients who did not achieve the endpoint were followed up until graft loss, lost to follow-up or death. Survival analysis was used to ascertain association between genetic markers, clinical data, and outcome. Fifty-three patients suffered a CE after renal transplantation. Results showed a significant association between *ANRIL* SNP and CE. Homozygous GG for the risk allele showed higher risk for CE than A carriers for the protective allele [HR = 2.93(1.69–5.11), $P < 0.0001$]. This effect was maintained when it was analyzed in combination with *CARD8*, suggesting that *CARD8* SNP could play a role in the *ANRIL* mechanism. However, our study does not clarify the molecular mechanism for the *CARD8* SNP regulation by *ANRIL*. *ANRIL* SNP may predispose to the development of CE after successful kidney transplantation.

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Key words
ANRIL gene, cardiovascular event, chronic kidney disease, renal transplant recipients, single-nucleotide polymorphism

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Introduction

Cardiovascular disease (CVD) is the leading cause of morbidity and mortality in kidney transplant recipients [1]. Approximately, 40% of patients have a cardiovascular event 3 years after transplantation [2]. Conventional risk factors for CVD, such as hypertension, diabetes mellitus, hyperlipidemia, obesity, and tobacco use, continue to be important in renal transplant recipients [3]. However, currently, it is accepted that the morbidity and mortality from CVD are not entirely considered by these conventional risk factors [3,4]. Time on dialysis,

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uremia, serum creatinine, anemia, alterations in the calcium and phosphate metabolism or even immunosuppression, especially calcineurin inhibitors, exacerbate the probability of developing diabetes, graft dysfunction, dyslipidemia, and hypertension, all of which increase cardiovascular morbidity [5–7].

Worldwide, stroke is one of the main causes of morbidity and mortality of cardiovascular disease [8], and it is caused by both genetic and environmental factors. Single-nucleotide polymorphisms (SNPs) at the 9p21 locus in chromosome 9 were found to be associated with both coronary artery diseases and ischemic stroke by genome-wide association studies (GWAS) [9,10]. The 9p21 locus contains a long noncoding RNA (LncRNAs), called *ANRIL* (Antisense Noncoding RNA in the INK4 Locus), which is believed to participate in gene regulation [11,12]. *ANRIL* SNPs have been associated with cardiovascular and all-cause mortality [13] and CVD morbidity in the general population. Different studies have described their participation in several pathologies such as myocardial infarction [14], coronary heart disease [15–17], ischemic stroke [18], or intracranial aneurysm susceptibility [19]. However, the information regarding chronic kidney disease (CKD) patients is limited. Until now, few studies have reported the role of *ANRIL* SNPs in CKD. Our previous work described, for the first time, a strong correlation between four *ANRIL* SNPs and major adverse cardiovascular events (MACE) in hemodialysis population [20]. Furthermore, the most representative polymorphism of the four *ANRIL* SNPs studied (rs10757278, rs4977574, rs10757274, and rs6475606) with the outcome analyzed in our population was *ANRIL* SNP rs10757278, corroborating the data described by Helgadottir *et al.* [14]. In the renal transplant field, only one study has confirmed the association of *ANRIL* SNPs with cardiovascular death [21].

Mechanisms by which *ANRIL* regulates downstream genes such as *CARD8* (caspase activation and recruitment domain 8) are not yet fully understood. However, it has been suggested that *ANRIL* SNP rs10757278 disrupts a binding site for the inhibitory transcriptional factor STAT1 [22]. The STAT1 signaling pathway mediates an inflammatory response through the stimulation of interferon gamma, and therefore, it has been suggested that *ANRIL* may play a role in inflammatory responses and atherosclerosis [23].

CARD8 encodes a component of innate immunity involved in the suppression of nuclear factor kB (NF-KB). This leads to the suppression of an immune response and inflammatory activity processes which otherwise occur through the NLRP3 inflammasome relationship [24]. The SNP rs2043211 of *CARD8* changes cysteine to a premature termination codon, thus yielding a truncated protein, which influences the protein function [25,26]. Previous investigations have studied the relationship of the *CARD8* SNP with different inflammatory diseases including cardiovascular disease: myocardial infarction [27], coronary atherosclerosis [27,28] and also with ischemic stroke [23]. However, conclusions are confusing and renal transplant recipients have never been studied in this context.

In the present study, we hypothesize that *ANRIL* SNP rs10757278 and *CARD8* SNP rs2043211 could mediate susceptibility to cardiovascular events in renal transplant recipients.

Materials and methods

Patient population and study design

This is an observational follow-up cohort study focused on the incidence of cardiovascular events and its correlation with genetic markers in renal transplant recipients.

The study was performed in accordance with the ethical standards laid down in the 2000 Helsinki Declaration and was approved by the Research Ethics Committee. All included patients gave written informed consent to take part in the study (PR 155/15). Furthermore, this study was conducted according to the "Strengthening the Reporting of Observational Studies in Epidemiology" guidelines.

A total of 1466 patients had kidney transplantation from January 2000 to December 2014 at Bellvitge University Hospital (Barcelona). Exclusion criteria for this study included loss of functioning graft before 3 months posttransplantation, multi-organ transplantation, or re-transplantation. Patients eligible but not recruited were those whose DNA was not available or with and incomplete clinical database. Furthermore, some patients were not assessed for eligibility because they missed invitation to participate or they declined to be invited. Finally, the study population consisted of 505 renal recipients. Patient data were retrospectively collected from patients' charts, and there were no missing data. All patients were treated with conventional immunosuppressive drug regimen consisting of oral tacrolimus, cyclosporine, or sirolimus in combination with mycophenolate mofetil. Induction therapy varied depending on the perceived immunological risk and

was also used for kidneys coming from extended criteria donors. Induction therapy, basiliximab, or daclizumab were used in the case of increased immunological risk. In addition, all patients received intra-operative glucocorticoids according to local protocol.

Definition of clinical variables

Demographic characteristics (age, gender, ethnicity, and body mass) of the patients were recorded the day of the renal transplant. The cause of end-stage renal disease (ESRD), dialysis status (predialysis, hemodialysis, or peritoneal dialysis), time on dialysis, hypertension, diabetes mellitus, smoking, pre-existence of cardiovascular disease, date of renal transplant, type of transplant (death donor or living donor), number of HLA-AB and HLA-DR mismatches, donor age and sex, cold ischemia time, and immunosuppressive treatment were also evaluated the day of the renal transplant.

Clinical outcome variables assessed as acute tubular necrosis, clinical acute rejection episodes, *de novo* diabetes mellitus, and graft loss were recorded after transplantation. Graft loss was defined as return to dialysis or re-transplantation. Furthermore, estimated glomerular filtration rate (eGFR) and cholesterol were recorded 3 months postrenal transplantation. eGFR was calculated using the CKD-EPI formula (Chronic Kidney Disease Epidemiology). Finally, proteinuria data (g/l) were collected a year after renal transplantation.

The endpoint of the study was defined as an occurrence of a cardiovascular event. For a deeper analysis, cardiovascular events were classified into two sub-types: ischemic stroke and myocardial event (comprising both myocardial infarction and unstable angina). A sub-study analyzing mortality as an endpoint was also developed. Mortality was classified as cardiovascular mortality and overall mortality.

All patients were followed up until the endpoint was achieved. The follow-up was stopped in case of graft loss, lost to follow-up, death, or the end of data collection, which was January 31, 2017.

SNP genotyping

Genomic DNA was extracted from peripheral blood of patients using the Wizard® Genomic DNA Purification Kit (Promega Corporation, Sydney, Australia) and was stored at −80 °C.

Genotyping of the *ANRIL* SNP rs10757278 and *CARD8* SNP rs2043211 was carried out using TaqMan SNP Genotyping Assay (assays ID: C_11841860_10 and C_11708080_1, respectively; Applied Biosystems, Foster City, CA, USA) in 384-well plates that included positive and negative controls. Real-time PCRs were carried out on the 7900HT Fast Real-time PCR System, Applied Biosystems (Thermo Fisher Scientific, Waltham, MA, USA), following standard recommendations. Briefly, 0.5 μl assay mix was mixed with 5 μl iTaq Universal Probes Supermix (part no. 172-5130), 1 μl genomic DNA (10–20 ng/μl), and 3.5 μl water (B. Braun, Barcelona, Spain). The resulting mixture was heated to 50 °C for 2 min and 95 °C for 10 min in the thermal cycler. This was then followed by 40 cycles of denaturization at 95 °C for 15 s and annealing/extending at 60 °C for 1 min. Samples were genotyped in CCiT (Centres Científics i Tecnològics) at University of Barcelona, Campus Bellvitge.

Statistical analysis

Descriptive analyses were performed to summarize the baseline data and the demographic characteristics for both transplant renal patients with and without a cardiovascular event.

Allele frequency distribution was tested for Hardy–Weinberg equilibrium. Linkage study between both SNPs (*ANRIL* rs10757278 and *CARD8* rs2043211) was performed using the Chi-square test.

Survival analyses consisted in a comparison by log-rank test of Kaplan–Meier survival curves stratified by genotypes. In addition, Cox's proportional hazard models were employed to analyze the relationship between SNPs and the time elapsed until each endpoint took place.

The covariance matrix estimates (VCE) were used to test for collinearity ($r > 0.4$). Finally, noncollinear covariates were kept to perform a backward stepwise analysis to build the final multivariate model.

On the other hand, the synergy of both SNPs was studied stratifying patients in three groups: carriers for the four risk alleles (GG *ANRIL* and AA *CARD8*), remaining carriers for both SNPs, and finally, all the other genotypes. Statistical calculations were performed with STATA 12.0 software. The level of significance was set at $P < 0.05$.

Results

Characteristics of the study population

A total of 505 adult renal transplant recipients from a deceased or a living kidney donor (mean age 51.9 ± 13.9 years) were included in the present study.

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Characteristics of the population stratified by cardiovascular event are displayed in Table 1. Warm ischemia time was under 45 min in all cases. The majority of renal transplant patients (87%) had panel reactive antibodies (PRA) lower than 10%.

All patients were treated with conventional immunosuppressive drug regimen consisting of a calcineurin inhibitor (CNI) [tacrolimus (71%) or cyclosporine (17%)] or mTOR inhibitor [sirolimus (11%) or everolimus (5%)] in combination with mycophenolate mofetil (92%). Additional antibody induction therapy was used in 92% of the patients, which consisted of anti-thymocyte globulin (ATG) (34%), basiliximab (51%), or daclizumab (7%).

At the moment of the renal transplantation, 86 patients were diabetic and the diabetic nephropathy was the main cause of end-stage renal disease in 78% of these patients. At the end of the study, 61 new patients developed diabetes mellitus (15%).

Genotyping results

Allelic frequencies of the SNPs investigated in the 505 recipients are shown in Table 2. Frequencies observed in the present study were in accordance with reported allele frequencies in a Caucasian population. All patients were of Caucasian ethnicity.

All investigated variants were highly prevalent. The frequencies of the AA, AG, and GG *ANRIL* genotypes were 22.6%, 50.7%, and 26.7%, respectively. The AA, AT, and TT *CARD8* genotypes were observed in 43.3%, 45.5%, and 11.2% of patients, respectively. When combining both *ANRIL* and CARD8 allelic statuses, the frequency of the GG *ANRIL* plus AA *CARD8* was 11.5%.

The genotyping data for *ANRIL* SNP rs10757278 and for *CARD8* SNP rs2043211 did not deviate from the Hardy–Weinberg equilibrium in our population (Table 2). A pairwise comparison using the exact test disequilibrium analysis yielded no indication of allelic dependence ($P = 0.59$) between our two SNPs in study.

ANRIL and *CARD8* SNPs and cardiovascular events

The follow-up period ranged from 3 months to 15.8 years, and the mean follow-up was 5.7 years. Throughout this period, 53 patients (10%) suffered a cardiovascular event (20 patients had an ischemic stroke and 33 patients had a myocardial event). The remaining patients ($n = 452$) were censored: 101 grafts were lost (44 returned to dialysis and 57 died with a functioning graft), seven patients dropped out, and 344 patients were cardiovascular event–free at the end of data collection. The first cardiovascular event occurred at an average period of 4.5 years after renal transplant.

Kaplan–Meier survival curve for *ANRIL* polymorphism rs10757278 showed statistical significance in the log-rank test comparing carriers of the protective allele (AA or AG) versus homozygotes for the risk allele (GG) (Fig. 1). Patients with the GG genotype showed a significantly higher risk of cardiovascular events than AA or AG ($P = 0.0003$). Additionally, we carried out a stratified analysis by type of cardiovascular event: ischemic stroke or myocardial event (myocardial infarction and unstable angina) (Figs 2 and 3). We observed that even though GG patients were significantly associated with both types of cardiovascular events, ischemic stroke ($P = 0.0008$) showed a stronger association with the *ANRIL* polymorphism rs10757278.

Relationship between cardiovascular events and *ANRIL* polymorphism was evaluated by univariate Cox regression analysis [HR = 2.65 (1.54–4.58), $P < 0.001$]. We found collinearity between eGFR CKD-EPI and gender ($r = -0.7458$), and between renal recipient age and donor age at renal transplantation ($r = -0.6493$). eGFR CKD-EPI and renal recipient age were introduced in the multivariate model, together with the noncollinear covariates, according to its stronger association with cardiovascular events.

After adjustment for covariates, the *ANRIL* polymorphism showed a significant relationship with cardiovascular events [HR = 2.93 (1.69–5.11), $P_{adj} < 0.0001$] (Table 3). Patients homozygous for the risk allele (GG) showed a 2.93-fold higher risk of suffering a cardiovascular event than patients carrying the protective allele (AA or AG genotype).

When cardiovascular events were studied separately (ischemic stroke and myocardial event), univariate Cox also showed a stronger association between *ANRIL* SNP and ischemic stroke [HR = 4.09 (1.67–9.99), $P = 0.002$] than with a myocardial event [HR = 2.02 (0.99–4.10), $P = 0.053$]. After adjustment for the covariates in the multivariate Cox analysis (Table 3), ischemic stroke [HR = 4.43 (1.81–10.85), $P_{adj} = 0.001$] and myocardial events [HR = 2.27 (1.10–4.67), $P_{adj} = 0.026$] remained significant.

Regarding *CARD8* polymorphism, no association was observed between *CARD8* SNP rs2043211 and cardiovascular events either in Kaplan–Meier curves (P log-rank test = 0.324) or in Cox univariate survival analysis [HR = 1.32 (0.76–2.30), $P = 0.326$].

To study the synergistic effect of *ANRIL* and *CARD8* polymorphisms, risk alleles were combined. Patients carrying the four risk alleles—*ANRIL* (GG)/*CARD8* (AA)—have

Table 1. Demographic and clinical data of all patients, cardiovascular event and cardiovascular event-free transplant recipients.

	All patients $n = 505$	Cardiovascular event-free $n = 452$	Cardiovascular event $n = 53$	*P* value
Cause of ESRD (%)				
Diabetes	13	12	25	0.150
Glomerular	27	27	26	
Interstitial	12	13	8	
Polycystic kidney disease	13	13	8	
Vascular	9	9	8	
Other	26	26	26	
Recipient sex (male) (%)	66.1	65.7	69.8	0.55
Recipient age (years)	51.9 ± 13.9	51.1 ± 13.9	57.9 ± 12.2	**0.0008**
BMI (kg/m^2)	25.7 ± 4.3	25.5 ± 4.1	26.7 ± 5.1	0.0536
Hypertension (%)	79.7	80.1	76.1	0.526
Smoke (%)				
Non	52	53	48	0.275
Yes	19	18	29	
Ex-smoker	29	30	23	
Diabetes mellitus pre-RT (%)	17.0	14.4	39.6	**<0.0001**
Dialysis time (years)	2.6 ± 3.0	2.5 ± 2.7	3.1 ± 4.4	0.2422
Dialysis status (%)				
Hemodialysis	81	79	94	0.240
Peritoneal	12	13	6	
Predialysis	7	8	0	
Cardiovascular disease pre-RT (%)	10.7	8.4	30.2	**<0.0001**
Donor sex (male) (%)	60.1	59.2	67.9	0.22
Donor age (years)	51.7 ± 16.5	51.8 ± 16.2	50.6 ± 18.7	0.5988
DD/LD	458/47	405/47	51/2	0.067
HLA-AB mismatches 0/1/2/3/4	20/27/161/206/91	16/24/146/185/81	4/3/15/21/10	0.5501
HLA-DR mismatches 0/1/2	107/325/73	97/290/65	10/35/8	0.6854
Cold ischemia time (min)	16.7 ± 7.8	16.5 ± 8.0	18.1 ± 5.1	0.1513
Acute tubular necrosis (%)	26.8	27.7	19.2	0.193
Clinical acute rejection (%)	17.7	17.5	19.6	0.73
eGFR CKD-EPI 3 months (ml/min/1.73 m^2)	48.5 ± 21.6	49.0 ± 22.0	44.3 ± 16.9	0.1318
Proteinuria one year (g/l)	0.45 ± 1.55	0.46 ± 1.62	0.42 ± 0.53	0.8921
Cholesterol 3 months (mmol/l)	4.35 ± 1.07	4.35 ± 1.09	4.30 ± 0.83	0.7636
Diabetes mellitus (%)	29.1	27.4	43.4	**0.016**

DD, deceased donor; ESRD, end-stage renal disease; LD, living donor; RT, renal transplant.

Continuous variables were expressed as mean ± SD (standard deviation). Categorical variables were expressed as percentages to the entire study patients. *P* values in bold were statistically significant in the Student *t* test or Chi-square test that compared cardiovascular event versus cardiovascular event-free cohort.

a 2.30-fold higher risk of suffering a cardiovascular event than patients carrying any other genotype combinations [HR = 2.30 (1.20–4.41), $P = 0.012$] as is shown in Fig. 4.

The stratified analysis showed a significant relationship only between *ANRIL*/*CARD8* SNPs and ischemic stroke [HR = 3.04 (1.17–7.91), $P = 0.023$]. Myocardial events did not achieve significance [HR = 1.85 (0.75–4.55), $P = 0.180$].

Moreover, the presence of the protective *CARD8* SNP rs2043211 T allele in the *ANRIL* SNP rs10757278 risk group (GG) seemed to counteract the effect of *ANRIL* SNP rs10757278 itself [HR = 2.06 (0.99–4.29), $P = 0.052$]. On the contrary, when the protective T allele of *CARD8* SNP rs2043211 was not present, the risk genotype GG of *ANRIL* maintained its statistical significance [HR = 2.67 (1.35–5.27), $P = 0.005$] reinforcing the protective role of *CARD8* T allele.

ANRIL and *CARD8* SNPs and exitus

The follow-up period ranged from 3 months to 15.8 years, and the mean follow-up was 6.0 years.

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Table 2. Genotype distributions of the polymorphisms (%) and *P* Hardy–Weinberg disequilibrium test.

	AA	AG	GG
ANRIL rs10757278 A/G			
All patients (*n* = 505)*	22.6	50.7	26.7
Cardiovascular event-free (*n* = 452)	22.8	52.9	24.3
Cardiovascular event (*n* = 53)	20.7	32.1	47.2
	AA	**AT**	**TT**
CARD8 rs2043211 A/T			
All patients (*n* = 505)**	43.3	45.5	11.2
Cardiovascular event-free (*n* = 452)	42.7	46.2	11.1
Cardiovascular event (*n* = 53)	49.1	39.6	11.3

P valuefor Fisher's exact test (Hardy–Weinberg disequilibrium test): **P* = 0.94 and ***P* = 0.95.

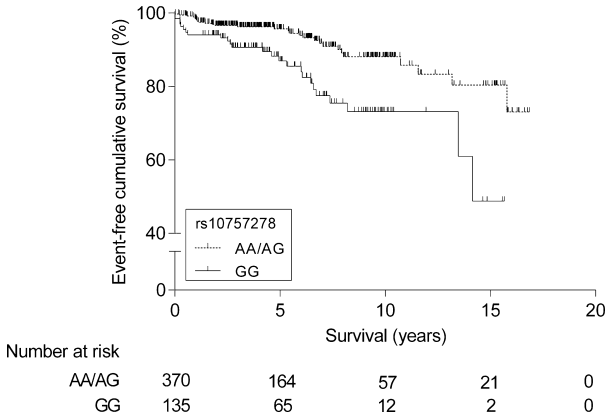

Figure 1 Kaplan–Meier survival curve for cardiovascular events for the *ANRIL* SNP rs10757278 AA/AG versus GG. Log-rank test, *P* = 0.0003.

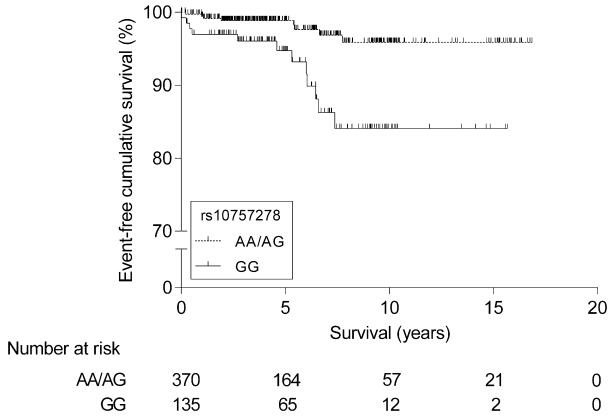

Figure 2 Kaplan–Meier survival curve for ischemic stroke for the *ANRIL* SNP rs10757278 AA/AG versus GG. Log-rank test, *P* = 0.0008.

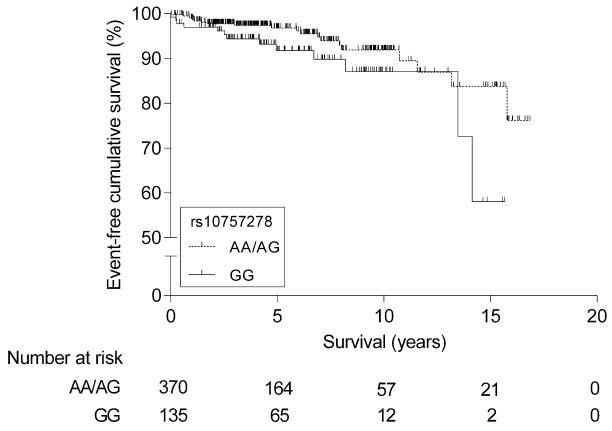

Figure 3 Kaplan–Meier survival curve for myocardial event for the *ANRIL* SNP rs10757278 AA/AG versus GG. Log-rank test, *P* = 0.048.

Throughout this period, 82 deaths (16%) were recorded, of which only 21 (4%) took place as a consequence of cardiovascular disease. During the follow-up, seven patients dropped out and 416 patients finished the data collection.

In univariate Cox analysis, no significant association was found between *ANRIL* SNP rs10757278 and all-cause mortality ($P = 0.139$) nor with cardiovascular exitus ($P = 0.761$). Furthermore, no association was observed between the *CARD8* SNP rs2043211 and all-cause mortality ($P = 0.155$) nor cardiovascular exitus ($P = 0.286$).

Discussion

ANRIL SNP has been related with a higher risk of developing cardiovascular events. This study explores its role in renal transplant patients. It analyzes the post-transplant incidence of cardiovascular events in a cohort of 505 renal transplant recipients, and it assesses the relationship between *ANRIL* and *CARD8* polymorphisms and the occurrence of a cardiovascular event. The incidence of cardiovascular events after renal transplant was 10% in our population. These results are in accordance with those of a previously published report by the US Renal Data System, where nearly 11–12% of a long cohort of patients had a cardiovascular event 3 years after transplantation [2]. Considering the fact that CVD is one of the major causes of death and disability in renal transplant patients, the identification of risk factors for CVD is crucial to prevent graft failure [4].

The comparison between Kaplan–Meier survival curves according to the genotype of the *ANRIL* SNP rs10757278 showed that a genetic variation in the

Table 3. Multivariate Cox survival analysis of cardiovascular event, ischemic stroke and myocardial event in transplant recipients.

	HR (95% CI)	*P* value
Cardiovascular event		
ANRIL rs10757278 (GG)	2.93 (1.69–5.11)	**<0.0001**
Recipient age (years)	1.06 (1.03–1.09)	**<0.0001**
BMI (kg/m^2)	1.05 (0.99–1.13)	0.122
Ischemic stroke		
ANRIL rs10757278 (GG)	4.43 (1.81–10.85)	**0.001**
Recipient age (years)	1.07 (1.02–1.11)	**0.002**
Myocardial event		
ANRIL rs10757278 (GG)	2.27 (1.10–4.67)	**0.026**
Recipient age (years)	1.06 (1.02–1.09)	**0.001**
BMI (kg/m^2)	1.06 (0.97–1.15)	0.217

BMI, body mass index; CI, confidence interval; HR, hazard ratio.

ANRIL SNP was introduced in the model as homozygous for the non-protective allele (GG) compared with carriers for the protective allele. *P* values in bold were statistically significant.

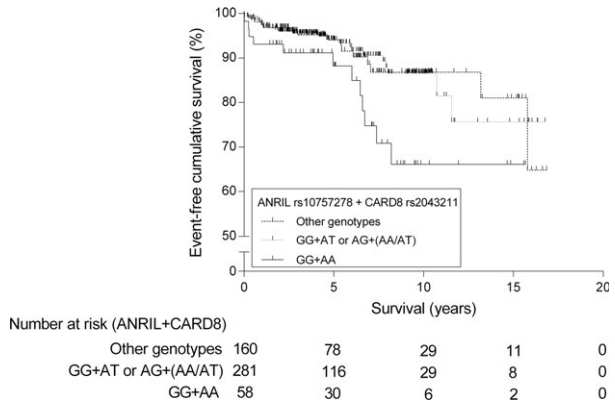

Figure 4 Kaplan–Meier survival curve for cardiovascular events for the synergy between *ANRIL* SNP rs10757278 and *CARD8* SNP rs2043211. Homozygous for both alleles (GG + AA), remaining carriers for the *ANRIL* SNP in combination with carriers for *CARD8* SNP (GG + AT, AG + AA/AT), and all other genotypes (GG+TT, AG + TT, AA + AA/AT/TT). Log-rank test, $P = 0.0360$.

ANRIL gene was associated with cardiovascular events in the whole cohort as well as in the general population [14–16]. Specifically, the variant GG allele in *ANRIL* polymorphism correlated with a high risk for a cardiovascular event to occur in renal transplant recipients. Among cardiovascular events, ischemic strokes showed the strongest association with *ANRIL* SNP rs10757278 in comparison with myocardial events. These results are supported by the univariate model for Cox regression where homozygous patients GG for *ANRIL* SNP rs10757278 showed a higher risk of a cardiovascular event occurring than AA or AG patients, confirming the findings of studies focusing on the general population [14–16,29–35]. Moreover, the hazard ratio was higher (HR = 4.09) when only ischemic stroke was considered compared with both multiple event types (HR = 2.65).

There are several studies showing that *ANRIL* is associated with increased risk of coronary atherosclerosis, carotid arteriosclerosis, peripheral artery disease, and other vascular diseases [36–39]. *ANRIL* is expressed in cells that play a critical role in atherogenesis, and although the molecular mechanism is not well established, it has been described that platelet reactivity and bone marrow megakaryopoiesis can be increased. Platelet production and activation may predispose to arterial thrombosis, suggesting an explanation, for the association of *ANRIL* SNP and cardiovascular events [40]. However, as a long noncoding RNA, *ANRIL* may play its role in atherosclerotic processes by influencing the expression of other genes such as *CARD8* [23]. It seems that the risk allele of *ANRIL* SNP rs10757278 disrupts a binding site for transcription factor STAT1 [22] mediating inflammatory responses.

Several epidemiological studies have identified factors associated with an increase of cardiovascular disease after kidney transplantation [41–43]. These studies suggest that the cardiovascular risk factors for the general population (e.g., old age, prior cardiovascular disease, diabetes, smoking, blood pressure, BMI, and glomerular filtration rates estimated with the CKD-EPI) are predictive of events in the transplant population. Moreover, it is shown that the episodes of acute rejection during the first year after transplantation are associated with a major risk [41,42]. Particularly, diabetes mellitus has been recognized as a major risk factor for atherosclerosis with a complex pathogenesis involving multiple biological processes. Moreover, immunosuppressant treatments, especially tacrolimus and steroids, have been associated with an increased risk of posttransplantation diabetes mellitus [44,45]. At the end of our study, 15% of recipients developed *de novo* diabetes mellitus. New-onset diabetes mellitus after transplantation occurs primarily in the first 3–6 months posttransplant and increases the risk of cardiovascular events, graft rejection and lessens the probability of patient survival [46,47].

In the current study, these risk factors were analyzed as covariates associated with cardiovascular events in the multivariate Cox analysis to minimize bias. We found association between the *ANRIL* SNP rs10757278 and cardiovascular events, mainly due to ischemic

stroke rather than myocardial infarction. In this sense, our results concluded that homozygous GG patients showed a 4.43-fold higher risk of an ischemic stroke event than patients carrying the protective A allele. These results are in agreement with those reported in the literature where the role of *ANRIL* SNP in ischemic stroke is well described. Recent meta-analysis [48,49] demonstrated that the rs10757278 SNP in *ANRIL* gene was a risk factor for developing ischemic stroke, particularly large-vessel strokes but not small-vessel or cardioembolic strokes. Our findings and previously reported studies suggest that *ANRIL* variants may exert more general effects on arterial wall function, such as vascular remodeling and/or repair, which is common in coronary heart disease and large-vessel stroke. However, there are other studies that did not find any correlation between *ANRIL* SNP and ischemic stroke [50,51]. The current study is the first that has found an association between ischemic stroke and cardiovascular events with *ANRIL* SNP rs10757278 in renal transplant recipients.

Furthermore, *ANRIL* regulates the expression of multiple genes, including *CARD8* [23]. Bai *et al.* showed that knockdown of *ANRIL* expression decreased *CARD8* expression and, on the contrary, overexpression of *ANRIL* increased *CARD8* expression. Moreover, *CARD8* is the only *ANRIL* downstream gene which has been proven to increase in expression in atherosclerotic lesions from carotid artery plaque tissue in renal transplant donors with ischemic cerebrovascular events [27]. Thus, we aimed to identify the influence of *CARD8* SNP rs2043211 on *ANRIL* SNP rs10757278 and also assess the association between *CARD8* SNP rs2043211 and cardiovascular events, ischemic stroke, and myocardial events in our population.

CARD8 negatively regulates nuclear factor KB activation, caspase 1-dependent interleukin-1beta secretion, and apoptosis, finally reducing the inflammatory response [52]. The *CARD8* SNP rs2043211 results in an A to T transversion that changes codon 10 into a stop codon in *CARD8* mRNA (Cys10Stop). Previous studies showed that homozygotes for the stop codon T allele could reduce the expression of *CARD8* and could impair the nuclear factor KB-inhibiting property of *CARD8* [53]. Paramel *et al.* [27] also showed that the minor allele was associated with lower expression of *CARD8* in the plaques, suggesting that low levels of functional *CARD8* protein may promote inflammation. On the contrary, carriers of the minor T allele of the *CARD8* SNP rs2043211 also displayed lower circulating C-reactive protein and lower levels of the pro-atherosclerotic chemokine MCP-1 [27]. Despite the fact that there are contradictory results regarding the risk allele [28] for the *CARD8* SNP rs2043211, our results are in concordance with other authors [23,27] reporting the T allele as the risk allele.

Regarding *CARD8* SNP, we did not find any association between the *CARD8* SNP rs2043211 and cardiovascular events, ischemic stroke, or myocardial events. These findings are not in concordance with Bai *et al.* [23] who demonstrate an association between the *CARD8* SNP rs2043211 and ischemic stroke in two independent Chinese Han populations. However, they did not find significant association with cardiovascular artery disease, in concordance with our results. On the other hand, Paramel *et al.* [27] did not find any significant association between the *CARD8* SNP rs2043211 and myocardial infarction in two independent cohorts. Moreover, in a Spanish cohort with rheumatoid arthritis, García-Bermúdez *et al.* [25] did not find any evidence for the role of *CARD8* SNP rs2043211 in the development of cardiovascular events.

Regarding the *ANRIL* SNP mechanism, considering that *ANRIL* and *CARD8* SNPs were not in linkage disequilibrium, the combined effect of *ANRIL* and *CARD8* SNPs was also studied. In this sense, patients carrying four risk alleles (*ANRIL* GG and *CARD8* AA) had a 2.30-fold higher risk of a cardiovascular event occurring than those patients who were carriers of any other genotype. In particular, the most important effect was found to be in relation to ischemic stroke (HR = 3.04). As *CARD8* alone did not show any relationship with cardiovascular events, it was expected that the combination of both SNPs would restrain the effect shown by *ANRIL* alone. However, the effect of the *ANRIL* SNP rs10757278 was maintained when *ANRIL* was analyzed in combination with *CARD8*, suggesting that *CARD8* SNP rs2043211 could play a role in the *ANRIL* SNP rs10757278 mechanism. This finding is in accordance with Bai *et al.* [23] who propose that *ANRIL* acts as an important modulator for expression of its downstream gene *CARD8*. The detailed mechanism by which *ANRIL* regulates *CARD8* expression remains to be identified but it has been suggested that long noncoding RNAs could have a novel role for Alu elements in epigenetic gene regulation [35]. This is the first study combining *ANRIL* and *CARD8* SNPs in renal transplant population. However, one potential limitation of our study is that it does not clarify the precise underlying molecular mechanism for the regulation of *CARD8* SNP by *ANRIL*. Furthermore, even though we do not suspect of any source of bias, our findings should be validated with an independent

cohort to confirm applicability to other renal transplant recipients' populations.

In summary, our findings suggest that the *ANRIL* SNP rs10757278 shows significant genotypic association with cardiovascular events, in particular with ischemic stroke, in renal transplant recipients. *ANRIL* could be used as a genetic marker to stratify patients according to their cardiovascular risk. These patients represent a high-risk group, and they should be followed carefully and probably treated more aggressively with statins and particularly with antiplatelet agents given the relationship between *ANRIL* and platelet reactivity in cardiovascular events complications. However, further studies are needed to demonstrate properly that such interventions can reduce the risk of cardiovascular events in patients carrying the *ANRIL* SNP rs10757278. An improved understanding of the pathogenesis of cardiovascular events would be beneficial for the management of cardiovascular risks in transplant recipients and for the potential clinical applications of these SNPs.

Authorship

AA-R, AP-M, MH, JMC and NL: designed research/study. AA-R, AP-M and PF: performed research/study. NL: contributed important reagents. AV-A, OB, IR, PA-R, JT, JMG and NL: collected data. AA-R, AP-M and NL: analyzed data. AA-R, AP-M, JMC and NL: wrote the paper.

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Conflict of interest

All authors have no financial or personal conflict of interest in relation to this article.

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