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ORIGINAL ARTICLE

Impact of NOD2/CARD15 haplotypes on the outcome after kidney transplantation

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Summary

Chronic allograft nephropathy and (cardiovascular) death with functioning graft are major causes of late graft loss. NOD2/CARD15 (nucleotide oligomerization domain-2/caspase-recruiting activating domain-15), an intracellular receptor, that is part of the innate immunity repertoire, has convincingly been shown to be involved in infection/inflammation-associated diseases. Specifically, NOD2/CARD15 polymorphisms are clearly associated with Crohn's disease and transplant-associated mortality after bone marrow transplantation. The aim of this study was to clarify the relevance of NOD2/CARD15-haplotypes in kidney transplantation. Three hundred fifty-two patients receiving their first kidney transplant were genotyped for the three major NOD2/CARD15 polymorphisms R702W, G908R and 1007fs with subsequent reconstruction of the different haplotypes. Four different NOD2/CARD15-haplotypes were observed in our population [CG(−): 89.8%, CGC: 3.5%, CC(−): 1.6%, TG(−): 5.1%). After stratifying the different haploypes into diplotypes (wild type: CG(−)/CG(−), $n = 284$, mutated haplotype, $n = 68$) we found a significant association with all-cause and cardiovascular mortality, also after adjusting to different covariates, and (only) in univariate analysis with graft survival. In conclusion, we found different effects of the NOD2/CARD15-haplotypes on disorders, like cardiovascular and all-cause mortality, which may be considered at least in part as chronic inflammation driven. Further studies are needed to confirm and work out the association between these disorders and the NOD2/CARD15-haplotypes.

Introduction

Long-term outcome after kidney transplantation has been nearly unchanged over the last 20 years but short-term survival has continuously improved. Therefore the etiology of chronic allograft nephropathy (CAN) as a major cause of long-term graft loss has come more and more in the focus of current research to better understand the process of chronic deterioration of allograft function.

In case of an acute rejection monocytes and T-effecter cells migrate into specific sites of the transplanted organ and produce a characteristic tubular and/or vascular infiltrate [1]. Similar mechanisms are involved in induction and maintenance of CAN. The adaptive and also the innate immune system are playing a crucial role in orchestrating inflammatory events such as acute rejection and/or CAN [2], and are also involved in other settings of inflammation, namely atherosclerosis [3] and viral/

bacterial infections in transplant recipients [4]. Beyond allograft rejection and infection, accelerated vasculopathy may also be observed within the graft and contribute to the faster decline in glomerular filtration rate. Pathophysiologically, these processes are closely connected, as evidenced by reports of e.g. infection-triggered rejection processes or rejection-mediated vasculopathy.

So far it has been assumed that allograft rejection is mainly mediated by the adaptive immune system, but there is increasing evidence that the innate immune system may also play an important role in the development of both acute and CAN [5,6]. A primary function of the innate immunity is the rapid recognition of so called danger signals, e.g. local presence of bacterial or viral proteins and nucleic acids, which enabled higher organisms to set up a first line of defense prior to an adaptive immune response. Furthermore, a role of innate immunity in the discrimination between self and foreign was suggested, which is particularly interesting in the setting of allotransplantation. The NOD2/CARD15 gene (nucleotide oligomerization domain-2/caspase-recruiting activating domain-15), which is a general (intracellular) receptor for both gram-positive and gram-negative bacteria and a member of the innate immune defense, is expressed in macrophages, expressed in atherosclerotic lesions, as well as on endothelial cells [7]. In biochemical and functional analyses muramyl dipeptide (MDP), the minimal motif of all peptidoglycans, has been identified as the essential structure recognized by NOD2/CARD15. These ligands are potent inducers of cytokine secretion in both monocytes and dendritic cells. Furthermore, these ligands are also known to induce the maturation of dendritic cells, as measured by increased expression of CD80, CD86 and MHC class II.

In large-scale genome wide studies a variety of single nucleotide polymorphisms (SNPs) in the NOD2/CARD15 were reported to be involved in the development of inflammatory bowel disease [8–10]. Within these different SNPs, three alleles, called SNP 8 (R702W), 12 (G908R) and 13 (1007fs or 3020insC, i.e. this is a frameshift mutation that leads to a Leucin to Proline substitution followed by a premature stop codon) have been identified to determine an increased susceptibility for Crohn's disease, dependent on the numbers of genetic variants from two to threefold for one up to 20–40-fold for two or more variants [8,11–15].

In several functional studies these three SNPs have been shown to lead to a reduction of nuclear factor κB (NFκB) activation (loss of function) in response to bacterial components [8,9,14,16]. However, in other studies evidence for gain of function in individuals with the described polymorphisms has been reported [17]. This difference might be explainable because of a different stimulation pathway in different cell types.

NFκB is an effective transcription factor in the secretion of pro-inflammatory cytokines, thus potentially leading to an iteration of signaling pathways of the innate immune system. This might suggest a role of NOD2/CARD15 polymorphisms in modulation of pro-inflammatory diseases, a fact that is supported by our own findings in a bone marrow transplant cohort [15]. Therein we could show a significant increase in transplant related mortality (TRM) due to the existing mutated polymorphisms. We found a graduation in TRM in relation to the source of mutation, i.e. the lowest cumulative incidence of TRM were seen in patients with no mutation within the three SNPs, with a rise if only the recipients had any mutated SNP, to the highest incidence if both recipient and donor NOD2/CARD15 expressed a mutated form. These results were confirmed in a subsequent multicenter study [18].

The major part of the TRM mentioned above is infection-associated, which is modulated by the NOD2/CARD15 polymorphisms. Therefore we postulated an impact of these polymorphisms on allograft and patient outcome (acute rejection, delayed graft function (DGF), cardiovascular events and infection) after renal transplantation due to a modulated inflammation response.

Patients and methods

Patient demographics

We consecutively included 352 patients receiving their first renal transplant at the transplantation center at the University of Regensburg between 1995 and 2006, comprising about 95.1% of all patients within that period; with a mean follow-up of 43.8 ± 31.3 months. Demographic data for donor and recipient age and sex, HLA-mismatch (HLA-MM), panel reactive antibodies (PRA), cold ischemia time (CIT), immunosuppressive therapy, presence of rejection episodes, laboratory values, clinical examinations and graft survival were extracted from the hospital records.

The standard immunosuppressive regimen included a calcineurin inhibitor (cyclosporin A or tacrolimus), a proliferation inhibitor (mycophenolate mofetil or azathioprine) and steroids. In cases of high immunological risk (e.g. high levels of PRA) induction by either polyclonal antithymocyte globulin (ATG) or monoclonal anti-CD25 antibodies was followed by a standard maintenance immunosuppressive treatment.

The Internal Ethical Review Board approved the study and written informed consent was obtained at the time of enrolment.

Determination of NOD2/CARD15-polymorphisms

Genomic DNA from allograft recipients was isolated from peripheral white blood cells using a standard salting out procedure. The different NOD2/CARD15 polymorphisms, namely SNP8 (R702W), SNP12 (G908R) and SNP13 (1007fs) were analyzed using the ABI Prism 7900HT Sequence Detection System® (Applied Biosystems, Darmstadt, Germany) by Taqman PCR according to published protocols [15].

Haplotype reconstruction, i.e. the alignment of the three polymorphisms on one DNA-strand, from population genetic data was performed using the PHASE STANDARD ANALYSIS software (version 2.1), as previously described [19]. Phase uses Gibbs sampling, which is a type of Marcov chain/Monte Carlo algorithm [20,21].

To examine the net effect of both of a patient's haplotypes on graft and cardiovascular outcome, we determined the patients' diplotype and, to enable reliable statistical testing with acceptable numbers per group, we formed two groups of diplotypes: group 1: CG(−)/CG(−), $n = 284$ (i.e. both haplotypes consist of the wild type alleles; (−) means no insertion of a C-base at position 3020); group 2: any other haplotype, $n = 68$ (i.e. any mutated polymorphism is grouped here). The fact, that susceptibility to Crohn's disease increase with the number of genetic variants within the NOD2/CARD15 gene, supports the choice for these diplotype groups [8,14].

Endpoints

Cardiovascular events

Myocardial infarction, malignant ventricular arrhythmias, acute cardiac failure or any cardiac intervention (PTCA +/− stent or revascularization), cerebral ischemia or intracerebral hemorrhage, peripheral amputation or intervention or death due to one of these events was defined as cardiovascular event.

Acute rejection episodes and DGF, graft survival, and renal function

Acute rejection episode was determined by allograft biopsy in >95% of cases or was defined by an increase in serum creatinine level by 30% or more from baseline, not attributable to other causes, with subsequent return to baseline after treatment with pulse steroids. Episodes of acute rejection were primarily treated with a pulse steroid therapy over 3 days, followed by an ATG treatment in steroid resistant cases. DGF was defined as need for dialysis within the first 7 days [22]. Graft survival was defined as recipient survival with a functioning renal transplant (i.e. censoring for death).

Glomerular filtration rate was estimated by the abbreviated formula of MDRD, as suggested by the K/DOQI Group [23].

Severe and urinary infection

Severe infection (within the 1st year) was assumed if bacterial or viral infections required hospitalization. Urinary infections (up to month 3) were assumed in case of a positive urinary culture or a significant count of urinary leukocytes with subsequent drop to normal values after treatment.

Analysis of cytomegalovirus (CMV) infection

CMV-load was routinely measured by PCR. The frequency of measurement varied depending on time after transplantation, from weekly in the first 4 weeks to every month or less thereafter. CMV infection was assumed if CMV-PCR was found positive (cut-off level 10^2–10^3 copies/ml). CMV risk was assumed if the donor was CMV IgG positive and the recipient CMV IgG negative. CMV prophylaxis with ganciclovir/valganciclovir was used in patients with a CMV risk constellation for a 3-month period post-transplant.

Statistical analysis

Results are expressed as mean ± SD, unless stated otherwise. Comparisons of continuous variables between groups were performed by nonparametric tests and of categorical variables by two-sided chi-squared or two-sided Fisher's exact test where applicable. Categorical variables were tested for potential confounding covariates (acute rejection, DGF, recipient age, gender, living donor, CIT, HLA mismatch, PRA, donor age or duration of dialysis) by uni- and multivariate logistic regression analysis. Survival analysis was performed by the Kaplan–Meier method comparing groups using the log-rank test, and by Cox-regression modeling.

The time variable was time from transplantation. Endpoints were graft survival, all cause mortality ('death with functioning graft') and cardiovascular death. To correct for different confounders we adjusted to the following covariates: cardiovascular event prior transplantation, diabetes mellitus, recipient and donor age, gender, donor type (living or cadaveric donor), CIT, acute rejection, DGF, duration of dialysis, and HLA-MM.

$P < 0.05$ was considered as statistically significant. Statistical analysis was performed with the SPSS® version 14.0 software package (SPSS Inc. Chicago, IL, USA).

Results

Demographic data of each genotype are presented in Table 1 and did not differ with respect to mean recipient

Table 1. Demographic and clinical data according to the different NOD2/CARD15-haplotypes at the time of transplantation.

Haplotype	Wild type $n = 284$	Mutated $n = 68$	*P*-value
Mean recipient age, years	50.4 ± 13.5	51.0 ± 15.2	0.58
Donor age, years	50.2 ± 16.0	50.8 ± 15.0	0.81
Gender (male), *n*	192 (67.6%)	48 (70.6%)	0.67
Living donor, *n*	66 (23.2%)	14 (20.6%)	0.75
CNI/proliferation inhibitor/steroids	284/271/278	68/64/67	0.98
Duration of dialysis, months	48.4 ± 33.9	47.6 ± 30.4	0.96
Patients on dialysis (prior TX), *n*	266 (93.7%)	63 (92.6%)	0.78
CIT, h	12.0 ± 7.7	11.8 ± 7.3	0.93
HLA-MM, *n*	2.6 ± 1.7	2.6 ± 1.9	0.85
PRA (%)	1.5 ± 9.0	1.4 ± 7.7	0.74
DGF, *n*	51 (18.0%)	9 (13.2%)	0.47
ARE, *n*	104 (36.6%)	20 (29.4%)	0.32
CVD (prior Tx), *n*	57 (20.1%)	16 (23.5%)	0.51
DM (prior Tx), *n*	48 (16.9%)	12 (17.6%)	0.86
AH (prior Tx), *n*	273 (96.1%)	63 (92.6%)	0.21
Hyperchol (prior Tx), *n*	117 (41.2%)	30 (44.1%)	0.68
CreaCl at 3 months (ml/min)	47.7 ± 19.9	43.7 ± 17.6	0.13
CreaCl at 6 months (ml/min)	47.8 ± 21.2	43.5 ± 18.5	0.12
Urinary infection, *n*	107 (27.7%)	22 (32.4%)	0.48
Severe infection, *n*	57 (20.1%)	13 (23.5%)	0.99
CMV infection, *n*	94 (33.1%)	29 (42.6%)	0.16
CMV risk constellation, *n*	67 (23.6%)	21 (30.9%)	0.22

CIT, cold ischemia time; HLA-MM, HLA mismatch; PRA, panel reactive antibodies; CNI, calcineurin inhibitor; ARE, acute rejection episodes; DGF, delayed graft function; CVD, cardiovascular disease; DM, diabetes mellitus; Hyperchol, hypercholesterolemia; CreaCl, creatinine clearance; AH, arterial hypertension.

age and gender, donor age, CIT, living donor, HLA-MM, PRA, immunosuppressive therapy, renal function at 3 and 6 months (creatinine clearance), occurrence of infection (urinary and severe infection), duration of dialysis or preemptive transplantation, CMV infection or CMV risk, presence of hypertension, hypercholesterolemia or diabetes mellitus at the time of transplantation, or cardiovascular disease prior to transplantation.

NOD2/CARD15 – gene polymorphism allele frequency and haplotype reconstruction estimation

Allele frequency distribution of the three SNP's are shown in Table 2. For all polymorphisms, our population did not deviate from Hardy–Weinberg equilibrium. The allele frequencies correspond closely to previously published data.

Results of haplotype reconstruction estimation are shown in Table 3. The observed frequencies are not different to the frequencies estimated by PHASE. Haplotype 5 (TGC, $n = 0$) was not estimated to be present in the cohort.

As it is biologically plausible to examine the net effect of both of a patient's haplotypes on outcome, we determined the patients' diplotype (Table 4), and to get reasonable amounts of different haplotypes we grouped them either to wild type [CG(−)/CG(−), $n = 284$] or not ($n = 68$).

Effect of haplotypes on the rate of acute rejection, DGF, graft survival and the occurrence of cardiovascular events

The overall incidence of acute rejection was 35.1% and 17.3% for DGF. For the different haplotypes, as well as

Table 2. NOD2/CARD15 allele frequency distribution.

	Wild type allele (homozygote)	Heterozygote	Mutated allele (homozygote)	Mutated allele frequency	*P**
SNP8 (R702W)	317 (90.1%, CC)	34 (9.7%, CT)	1 (0.3%, TT)	0.05	0.6
SNP12 (G908R)	341 (96.6%, GG)	11 (3.1%, GC)	0 (CC)	0.03	1.0
SNP13 (1007fs)	325 (93.1%, no ins)	23 (6.6%, no ins/ins)	1 (0.3%, ins/ins)	0.03	0.4

*Pearson's chi-squared test for deviation from Hardy–Weinberg equilibrium.

Table 3. Haplotype reconstruction estimation using phase software (version 2.1).

Haplotype number	Allele at SNP8	Allele at SNP12	Allele at SNP13	Estimated frequency	Standard error	Observed frequency
1	C	G	–	0.899	0.001	0.898
2	C	G	C	0.036	0.0006	0.035
3	C	C	–	0.015	0.001	0.016
4	T	G	–	0.050	0.001	0.051
5	T	G	C	0.0009	0.001	0

Table 4. NOD2/CARD15 gene diplotype frequency.

NOD2/CARD15 diplotype	
1-1	284 (80.7%)
1-2	23 (6.5%)
1-3	9 (2.5%)
1-4	32 (9.1%)
2-2	1 (0.3%)
3-4	2 (0.6%)
4-4	1 (0.6%)

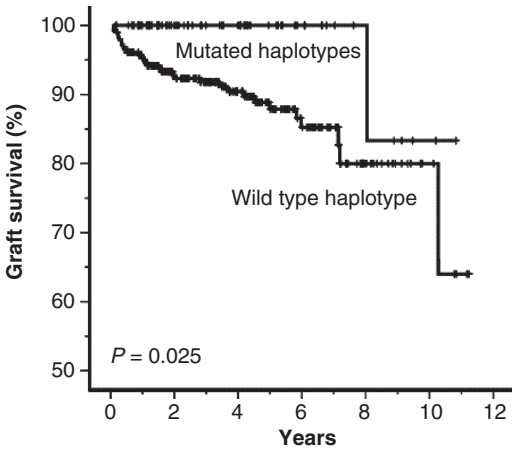
for all individual polymorphisms except the SNP13 ($P < 0.05$ for acute rejection), we found no significant association with acute rejection or DGF.

There was no effect of any single polymorphism on graft survival or occurrence of cardiovascular events/deaths (data not shown). However, after haplotype reconstruction and grouping the determined patients' diplotype into two groups (wild type versus any mutated haplotype), there was a significant effect for a better graft survival for carriers of the mutated haplotype ($P = 0.025$, Fig. 1). In the multivariate analysis this finding failed marginally ($P = 0.058$, Table 5).

Interestingly, for the overall graft survival (without censoring for 'death with functioning graft', i.e. loss of graft or death was counted as graft failure) we could not find any association with the different haplotypes (data not shown).

On the other hand, we observed a significant association of the two different diplotype groups (any mutated haplotypes versus wild type haplotype) with all cause mortality ('death with functioning graft'), with a significant higher rate of deaths in the group with the mutated haplotypes (group 2) (Fig. 2). In a further in-depth analysis we found that about 50% of these patients dying with a functioning graft have had a fatal cardiovascular event.

This is also reflected by a significant higher incidence of cardiovascular deaths (Fig. 3) in patients with any mutated haplotype. Both associations ('all cause mortality' and 'cardiovascular death') remain significant after adjusting to different confounders.



Patients at risk

Year	0	1	2	3	4	5	6	7	8	9	10
Wild type	284	244	190	160	123	90	64	36	19	11	6
Mutated	68	57	41	31	27	19	13	8	6	4	2

Figure 1 Kaplan–Meier estimate of graft survival in renal transplant recipients and numbers at risk with respect to the NOD2/CARD15 (nucleotide oligomerization domain-2/caspase-recruiting activating domain-15) haplotype. The presence of a mutated NOD2/CARD15 haplotype is associated with a significantly higher rate of graft survival (log rank $P = 0.025$).

Furthermore, the incidence of cardiovascular events was not significantly different between the two groups (data not shown).

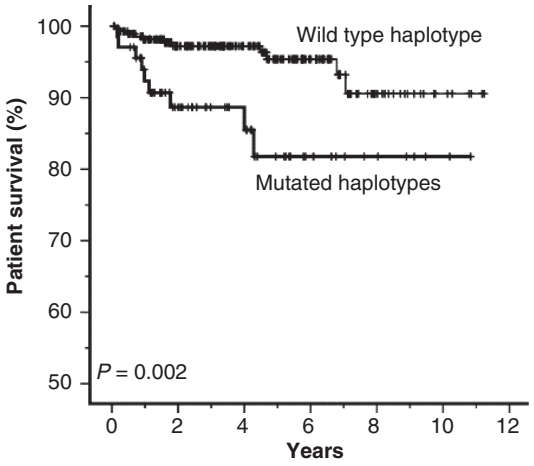
Discussion

The NOD2/CARD15 wild type (CG(−)/CG(−) haplotype (group 1) seems to have an unfavorable effect on the outcome of graft survival ($P = 0.025$, Fig. 1), though this difference is no longer significant in the multivariate analysis ($P = 0.058$, Table 5). In a further in-depth analysis, it was surprising that in the group with a mutated haplotype (group 2) only one graft loss (due to tumorous infiltration) within the observational period occurred. However, in both groups almost the same number of patients died with a functioning graft, despite much less patients being in group 2. Therefore, when we analyzed the all cause mortality in our cohort, i.e. death with functioning graft, we found a significant benefit for carriers of the wild type haplotype in group 1, [CG(−)/CG(−)] ($P = 0.002$, Fig. 2), which is also seen after correction for different known risk factors ($P = 0.002$, Table 5). In another in-depth analysis, we found that the majority of these deaths occurred by a fatal cardiovascular event ('cardiovascular death'), which is also reflected by a significantly higher rate of cardiovascular deaths in patients carrying at least one mutated haplotype (group 2)

Table 5. Cox-proportional hazard analysis to assess the effect of the NOD2/CARD15-haplotype on death with functioning graft (multivariate analysis with different models).

	'Graft survival'		'All cause mortality' ('death with functioning graft')		'Cardiovascular death'	
	P-value	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value	HR (95% CI)
Haplotype (ref. wildtype)	0.058	6.93 (0.94–51.22)	**0.002**	**0.21 (0.08–0.55)**	**0.004**	**0.05 (0.01–0.38)**
CV event before Tx (ref. no)	0.806	0.88 (0.33–2.40)	**0.040**	**3.00 (1.05–8.56)**	0.079	6.36 (0.81–50.26)
DM (ref. no)	0.420	0.69 (0.28–1.71)	**0.036**	**5.07 (1.12–23.05)**	0.118	4.77 (0.67–33.76)
Recipient age (age)	0.907	1.00 (0.97–1.04)	**0.002**	**1.13 (1.05–1.23)**	0.118	1.13 (0.97–1.32)
Gender (ref. male)	0.401	0.70 (0.31–1.61)	0.171	0.40 (0.11–1.49)	0.962	
Living donor (ref. cadaveric donor)	0.554	0.63 (0.13–2.97)	0.684	1.70 (0.13–21.96)	0.978	
CIT (hours)	0.239	1.05 (0.97–1.13)	0.827	1.01 (0.91–1.13)	0.788	1.02 (0.87–1.19)
AR (ref. no)	**0.004**	**3.16 (1.46–6.87)**	0.784	1.15 (0.42–3.13)	**0.021**	**9.72 (1.40–67.50)**
DGF (ref. no)	0.157	1.79 (0.80–4.01)	**0.006**	**4.95 (1.60–15.38)**	**0.002**	**32.87 (3.69–292.73)**
HLA-MM (number)	0.140	1.21 (0.94–1.55)	0.838	1.03 (0.75–1.42)	0.929	0.98 (0.57–1.66)
Donor age (years)	0.185	1.02 (0.99–1.05)	0.687	1.00 (0.97–1.04)	0.816	1.00 (0.95–1.07)
Duration of dialysis (years)	0.904	1.00 (0.90–1.01)	0.372	0.99 (0.97–1.01)	0.710	1.01 (0.98–1.038)

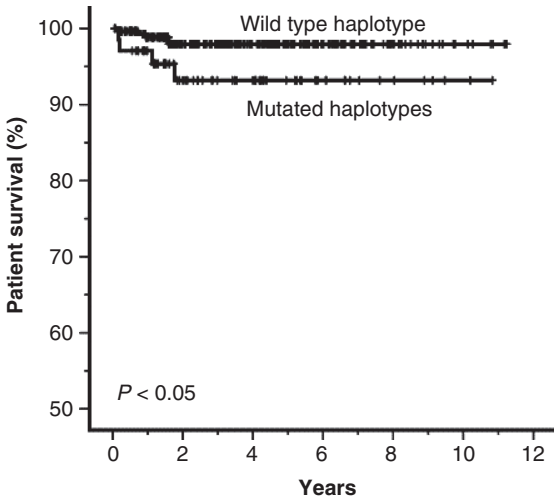
HR, hazard ratio; CI, confidence interval; CV event, cardiovascular event; DM, diabetes mellitus; CIT, cold ischemia time; AR, acute rejection; DGF, delayed graft function; HLA-MM, HLA mismatch. Bold figures highlight significant levels ($P<0.05$).



Patients at risk

Year	0	1	2	3	4	5	6	7	8	9	10
Wild type	284	244	190	160	123	90	64	36	19	11	6
Mutated	68	57	41	31	27	19	13	8	6	4	2

Figure 2 Kaplan–Meier estimate of death with functioning graft in renal transplant recipients and numbers at risk with respect to the NOD2/CARD15 (nucleotide oligomerization domain-2/caspase-recruiting activating domain-15) haplotype. The presence of a mutated NOD2/CARD15 haplotype is associated with significantly higher rates of death with functioning graft (log rank $P = 0.002$).



Patients at risk

Year	0	1	2	3	4	5	6	7	8	9	10
Wild type	284	244	190	160	123	90	64	36	19	11	6
Mutated	68	57	41	31	27	19	13	8	6	4	2

Figure 3 Kaplan–Meier estimate of cardiovascular death in renal transplant recipients and numbers at risk with respect to the NOD2/CARD15 (nucleotide oligomerization domain-2/caspase-recruiting activating domain-15) haplotype. The presence of a mutated NOD2/CARD15 haplotype is associated with significantly higher rates of cardiovascular death (log rank $P < 0.05$).

($P < 0.05$, Fig. 3). Interestingly no association with the two NOD/CARD15 haplotype groups could be observed for the occurrence of cardiovascular events, the incidence of acute rejection, DGF or major infective complications.

Similarly to the findings of Courivaud *et al.* [24], who reported no association between NOD2/CARD15 gene polymorphisms and atherosclerotic events after renal transplantation, we found no association between the different endpoints and each single polymorphism. On the other hand Courivaud *et al.* [24] did not report results of haplotype reconstruction in their paper. Furthermore, it

has to be mentioned, that Courivaud *et al.* [24] collected their patients in a totally different manner. In our cohort, almost every patient ever transplanted at our center, over 95%, was enrolled, therefore we have no selection bias due to early graft loss, death or loss to follow-up, as it would be proposed in the population mentioned above, which was collected in the outpatient clinic 1 year after transplantation. It is reasonable that patients with a worse course after transplantation in the study of Courivaud *et al.* [24] were lost to follow-up due to premature loss of graft function or patient death. Therefore, it is difficult to directly compare both studies.

The NOD2/CARD15-polymorphisms, initially known because of their impact in inflammatory bowel disease, seem to diminish NFκB activation in response to pathogen-associated molecular patterns (PAMPs) [9]. However, Pauleau *et al.* [25] found in NOD2/CARD15-deficient mice that NOD2/CARD15 deficiency did not affect the response of macrophages to multiple toll-like receptor agonists in terms of NFκB target activation, MAPK activation, and cytokine secretion. Nevertheless, these NOD2/CARD15-deficient mice were significantly protected when exposed to high systemic endotoxin and the macrophages were refractory to MDP stimulation. Furthermore, the NOD2/CARD15 expression is not limited to epithelial cells, granulocytes, dendritic cells or macrophages/monocytes at all, but the receptor is also expressed on endothelial cells [7,26]. The endothelium is one major target of PAMP-induced events, which are accountable for local inflammation which might be responsible for chronic vascular alterations, e.g. atherosclerosis or CAN. Up to now, there is a controversial discussion about the effects in the literature, i.e. loss or gain of function, of these NOD2/CARD15 polymorphisms, especially outside the gut [17]. Our data support on the one hand the hypothesis of loss of function, because patients with an altered expression of the NOD2/CARD15 receptor never lost their graft due to CAN, but on the other hand most of these patients died due to a cardiovascular event or a malignant disease, which might implicate a gain of function due to these polymorphisms. This hypothesis goes along with our findings on cardiovascular complications/deaths, which were more often seen in patients with an altered receptor being more consistent with a gain of function. This result has to be seen with caution due to the low number of events, but it was consistent in the multivariate adjustment. These findings go along with the setting in inflammatory bowel disease, in which the loss of function of the NOD2/CARD15-receptor sustains chronic inflammation, maybe by mechanisms that are similar to those underlying chronic inflammation that results in atherosclerosis. It is conceivable, that an altered NOD2/CARD15-receptor function leads to an impaired elimination of (intestinal) 'danger signals', and leads therefore to a higher inflammatory status. Furthermore direct involvement of an impaired monocyte/macrophage or even endothelial response in the vessels might be an alternative explanation and should be addressed in careful analyses in future studies. Clinically, these data seem to support altered inflammatory cascades that are also seen in other inflammation-prone settings. In bone marrow transplantation, we could also show the emerging role of these NOD2/CARD15 polymorphisms. The TRM was increased in the presence of any mutated NOD2/CARD15-receptor. Interestingly, we found also a graduation in TRM in relation to the source of mutation, i.e. the lowest cumulative incidence of TRM were seen in patients with no mutation within the three SNPs, with a rise if only the recipients had any mutated SNP, to the highest incidence if both recipient and donor NOD2/CARD15 expressed a mutated form of the NOD2/CARD15 gene [15,18].

In conclusion, we found in our studies suggestive associations of the NOD2/CARD15 haplotypes with disorders, like cardiovascular and all-cause mortality, and to a lesser degree with graft failure, which may be considered at least in part as chronic inflammation driven. Further studies are needed to confirm and work out the association, and the underlying mechanisms between both cardiovascular deaths and graft survival, and NOD2/CARD15 polymorphisms.

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