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**NO EVIDENCE OF ASSOCIATION BETWEEN NOD2/CARD15 GENE  
POLYMORPHISM AND ATHEROSCLEROTIC EVENTS AFTER RENAL  
TRANSPLANTATION<sup>1</sup>**

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## Footnotes

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**Abbreviations:**

AE: atherosclerotic events; CI: confidence interval; CVD: cardiovascular disease; GVHD: graft-versus-host disease; HR: Hazard ratio; NOD2/CARD15: Nucleotide Oligomerisation Domain-2/Caspase-Recruitment Domain-15; PCR: polymerase chain reaction; RR: relative risk; RTR: renal transplant recipient; SNP: single nucleotide polymorphism; TLR4: Toll-like receptor-4

## **Abstract**

Stable renal transplant recipients (RTR) display high rates of atherosclerotic events (AE). Innate immunity and especially vascular inflammation play a role in the pathogenesis of atherosclerosis. It is illustrated both by an increased occurrence of post-renal transplant cardiovascular events in patients with elevated levels of C-reactive protein and by a correlation between post-transplant AE and Toll-like receptor-4 Asp299Gly polymorphism. Here, we analyze the influence NOD2/CARD15 gene polymorphism since NOD2 can modulate macrophage pro-inflammatory activity and macrophage is present in early atherosclerotic lesions. The incidence of single nucleotide polymorphism (SNP) in the three major polymorphic region of NOD2 gene (SNP8, SNP12 and SNP13) was assessed in 182 RTR and the correlation between such polymorphism and the development of AE was analyzed. No correlation was observed between NOD2 gene polymorphism and the occurrence of AE after renal transplantation. NOD2 gene polymorphism thus does not appear to influence cardiovascular complications in RTR.

Stable renal transplant recipients (RTRs) display disproportionately high rates of atherosclerotic events (AE) (1). It is now established that both vascular inflammation and components of the immune system are involved in the pathogenesis of atherosclerosis (2). We have already shown the importance of inflammatory and innate immunity in the pathogenesis of atherosclerosis after renal transplantation by demonstrating a correlation between the occurrence of post-transplant cardiovascular events and i) elevated circulating levels of C-reactive protein (3) and ii) Toll-like receptor-4 (TLR4) Asp299Gly polymorphism (4). Here, we analyze NOD2/CARD15 (Nucleotide Oligomerisation Domain-2/Caspase-Recruitment Domain-15, thereafter called NOD2) gene polymorphism. Three major single nucleotide polymorphisms (SNP) have been reported in NOD2 gene; SNP8, 12 and 13 alleles have been previously associated with increased susceptibility for two inflammatory diseases: Crohn's disease (5,6) and Graft-versus-Host Disease (GVHD) after allogeneic hematopoietic cell transplantation (7). This suggests that NOD2 gene polymorphism modulates pro-inflammatory diseases. NOD2 is expressed in macrophages (8,9) and macrophages are present in atherosclerotic lesions at early stages (10). To date, no study has evaluated the impact of NOD2 gene polymorphism on AE after transplantation. We assessed the frequency of NOD2 gene polymorphism in 182 RTRs and analyzed the relationship between such polymorphism and the development of atherosclerotic complications.

One hundred and eighty-two RTRs were enrolled in this trial. These patients have been transplanted between January 1990 and December 2000. Age, gender, body mass index (weight [Kg]/size<sup>2</sup> [m<sup>2</sup>]), past history of cardiovascular disease (CVD) and other risk factors were collected at transplant time (TABLE 1). Mean age was  $46 \pm 13$  years, and 117 RTRs (64.3 %) were men (TABLE 1). Mean follow-up of these 182 patients was  $7.5 \pm 2.3$  years post-transplant. These 182 RTRs were genotyped for SNP8, SNP12 and SNP13 of NOD2 gene. Genomic DNA was extracted from white blood cells using standard salting out procedure. Analysis of each NOD2 variants was then performed using a polymerase chain

reaction (PCR)-based genotyping assay with specific primers, as described (5). After PCR amplification of the polymorphic regions of interest, PCR products were digested overnight with Msp I, Hha I and ApaI restriction endonuclease (New England Biolabs, Beverly, USA), for SNP8, SNP12 and SNP13 respectively (5), as recommended by the supplier. Digestion products were then separated by a 2% standard agarose gel electrophoresis (Fig. 1). For each PCR, a negative control (PCR amplification without genomic DNA) was included. We added systematically a mutated DNA to control digestion procedures. Researchers and laboratory staff did not have any access to identifiable information and could identify samples by number only.

Post-transplant AE, i.e., coronary heart disease, stroke/cerebrovascular disease and abdominal aortic or lower extremity arterial disease, were diagnosed according to published criteria (11). Thirty-eight (20.7%) patients presented AE: 19 coronary heart diseases, 8 cerebrovascular diseases and 11 lower extremity arterial diseases. Statistical analysis was performed using Statview 5 (SAS institute Inc., Cary, NC). Arithmetic mean was calculated and expressed as  $\pm$  standard deviation (SD). Dichotomic variables were compared with the  $\chi^2$  test and continuous variables with the Student t test. Using log-rank tests on Kaplan Meier nonparametric estimates of the survival without AE distribution, we selected variables with a p value lower than, or equal to, 0.20. The selected variables were included into a Cox proportional hazard model, and a backward stepwise selection process was performed, this time at a classical  $\alpha = 0.05$ .

The incidence of heterozygous genotype carriers observed in the RTR cohort was limited: 8.8% (n=16), 4.4% (n=8) and 1.6% (n=3) for SNP8, SNP12 and SNP13, respectively. NOD2 allele and genotype frequencies are summarized in TABLE 2. As previously described (5,6), we did not find either heterozygous composites or homozygous mutants (TABLE 2). We observed more SNP12 heterozygote patients (4.4%; TABLE 2) than in a previous publication reporting SNP12 incidence (0.72 and 1.23%) in two independent Caucasian populations (6).

We did not find any relationship between NOD2 gene polymorphism and major complications observed after renal transplantation, including: acute rejection, CMV disease, bacterial or opportunistic infections, and death (TABLE 1). When focusing on occurrence of AE after renal transplantation, no correlation was also found (hazard ratio, HR, 1.12; 95% confidence interval, CI, 0.69 to 2.01;  $p=0.31$ ) (TABLE 1&2). In multivariate analysis, older age (HR, 4.77; 95% CI, 2.37 to 9.80), a past history of cardiovascular disease (relative risk, RR, 3.20; 95% CI, 1.49 to 6.45), male gender (HR, 1.99; 95%CI, 0.99 to 3.78); and diabetes mellitus (RR, 1.48; 95% CI, 1.11 to 2.75) predicted independently the risk of AE. This contrasts with the implication of NOD2 gene polymorphisms in other inflammatory diseases such as Crohn's disease (5,6) and GVHD (7). Several limitations should however be considered. The population studied was limited with regards to the frequency of polymorphism. However, the total number of patients was not very different from a previous publication associating NOD2 gene polymorphism and Crohn's disease occurrence (6). Furthermore, study design leads to include patients with a low CVD risk. However, with nearly the same cohort, we had previously shown that RTRs with TLR4 Asp299Gly polymorphism exhibited a lower risk of post-transplant AE (4). This led us to discuss the respective roles of NOD2 and TLR4 in pathogen recognition. NOD2, like TLRs, belongs to a large family of proteins involved in infectious pathogen recognition (12). As known for TLR4 at the cell membrane, intracellular NOD2 protein has been proposed to play a role in the intracytoplasmic response to bacterial cell wall products (muramyl dipeptide) and subsequent NF- $\kappa$ B activation in macrophages (12). In our previous study (4), RTRs with Asp299Gly TLR-4 polymorphism were less likely to experience post-transplant AE (RR=0.44), but developed more frequently severe bacterial infections (RR=1.33) and opportunistic infections (RR=3.03). These previous data together with the present data (i.e., absence of correlation between a particular NOD2 gene polymorphism and post-transplant AE occurrence) could support the idea that bacterial infections do not play a major role in the development of atherosclerosis. The relationship

between infectious pathogens and atherosclerosis, originally suggested by seroepidemiologic studies (13), was also confirmed by a series of investigations demonstrating the presence of pathogens in atherosclerotic lesions (14). On the other hand, transient prophylactic antibiotic treatment did not prevent secondary coronary syndromes (15,16). Therefore, the etiologic or pathogenic significance of bacterial infections in CVD remains to be determined. Finally, the functional role of NOD2 is still unclear. Most studies which implicated NOD2 in pro-inflammatory NF- $\kappa$ B pathway in macrophages were performed *in vitro* (8,9). They suggested that the association of NOD2 mutations was characterized by a diminished NF- $\kappa$ B response (8,9). However, the first functional study performed *in vivo*, using C57BL/6 mice deficient for NOD2 gene (NOD2<sup>-/-</sup> mice), did not confirm such *in vitro* results (17). Macrophages from NOD2<sup>-/-</sup> mice responded normally to TLR ligands in terms of NF- $\kappa$ B activation and inflammatory cytokine production (17). These results suggest the absence of NOD2 implication in NF- $\kappa$ B activation in macrophage. A more recent study using NOD2<sup>-/-</sup> mice reported that NOD2 was especially critical to regulate innate bacterial immunity in the intestinal tract (18). This was attested by an increased susceptibility of NOD2<sup>-/-</sup> mice to intragastric infection with *Listeria monocytogenes* but not after intravenous or intraperitoneal injection of these bacteria (18). NOD2 is highly expressed in crypts from the terminal ileum (18) in Paneth cells (19). This may explain why NOD2 mutations render patients susceptible to Crohn's diseases, a chronic inflammatory bowel disease (5,6) and GVHD (7). It is believed that the loss of gastrointestinal tract integrity, and the translocation of endogenous endotoxins into the systemic circulation, is a critical event in the initiation of GVHD (20). Overall, NOD2 mutations may not have a major influence of vascular inflammation and this may account for the results we are reporting here. Lastly, one has to consider the potential influence of NOD2 and TLR4 ligands. TLR4 ligands are generally stronger NF- $\kappa$ B activators than NOD2 ligands (12). In addition to pathogens, a number of endogenous ligands (e.g., heat shock proteins known to be present in atherosclerotic lesions, extracellular matrix proteins,...) have been

shown to stimulate TLR4 (12). This again may explain why we found a relationship with TLR4 Asp299Gly polymorphism while not with NOD2 gene polymorphism.

In conclusion, NOD2 gene polymorphism is not associated with AE in RTRs population and should not be proposed to assess CVD risk in RTRs. These results underline recent data showing that bacterial infections are not involved in CVD (15,16) and the major role of NOD2 is to control intestinal inflammation (18).

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**TABLE 1. Comparison of baseline characteristics and main outcome after transplantation in 182 renal transplant recipients (RTR) presenting a wild type genotype of the NOD2 gene versus RTRs presenting a mutation in SNP8, SNP12 or SNP13 of the NOD2 gene.**

	Wild type genotype carriers n = 155 (85.2%)	NOD2 polymorphism carriers <sup>a</sup> n = 27 (14.8%)
Age (years)	45.8 ± 13.8 <sup>a</sup>	49.4 ± 9.2 <sup>a</sup>
Sex ratio (Male / Female)	97 / 58 (62.6% / 37.4%)	20 / 7 (74% / 26%)
Pre-transplant Body Mass Index (kg/m <sup>2</sup> )	22.6 ± 4.1 <sup>b</sup>	24.3 ± 4.2 <sup>b</sup>
Past history of cardiovascular disease	8 (5.1%)	0
Hypertension	85 (54.8%)	17 (62.9%)
Dyslipidaemia	61 (39.3%)	10 (37%)
Diabetes mellitus (DM) / post-transplant DM	8 (5.2%) / 18 (11.6%)	1 (3.7%) / 5 (18.5%)
Homocysteine (µmol/l)	16.6 ± 5.5 <sup>a</sup>	15.2 ± 6.7 <sup>a</sup>
Cigarette smoking	15 (9.7%)	5 (18.5%)
Atherosclerotic events after transplantation	32 (20.6%)	6 (22.2%) <sup>c</sup>
Opportunistic infections	39 (25.2%)	5 (18.5%)
CMV disease	38 (24.5%)	5 (18.5%)
Bacterial infections	55 (35.5%)	7 (25.9%)
Acute rejection	30 (19.3%)	6 (22.2%)
Death after transplantation	5 (3.2%)	2 (7.4%)

Results are expressed in Kg/m<sup>2</sup> for body mass index, in µmol/l for homocysteine, in years for age and for other items as number of patients and percentage in brackets.

<sup>a</sup> heterozygote RTRs with a mutated NOD2 allele in either SNP8, SNP12 or SNP13 region (since no heterozygous composites or homozygous mutants were detected); <sup>b</sup> mean ± SD; <sup>c</sup> 2 patients with a mutated SNP8, 2 with a mutated SNP12 and 2 with a mutated SNP13 developed AE (see also TABLE 2).

**TABLE 2. Comparison of NOD2 gene polymorphism incidence in renal transplant recipients (RTR) enrolled in our cohort with three previously published cohorts including patients suffering from Crohn’s Disease**

		polymorphism incidence in RTR (n=182)	AE in these RTR (n=38)	polymorphism incidence in Ref. 5 (n=300)	polymorphism incidence in Ref. 6 (n=373)	polymorphism incidence in Ref. 6 (n=202)
SNP8	WT	166 (91.2%)	36 (21.7%)	289 (96.3%)	95.2%	97.2%
	H/h	16 (8.8%)	2 (12.5%)	11 (3.7%)	4.8%	2.8%
	DM	0	0	0	0	0
SNP12	WT	174 (95.6%)	36 (20.7%)	300 (100%)	99.3%	98.8%
	H/h	8 (4.4%)	2 (25%)	0	0.7%	1.2%
	DM	0	0	0	0	0
SNP13	WT	179 (98.4%)	36 (20.1%)	290 (96.7%)	95.9%	98.8%
	H/h	3 (1.6%)	2 (66.6%)	10 (3.3%)	4.1%	1.2%
	DM	0	0	0	0	0

Results are expressed as number of patients and/or percentage.

Abbreviations used: WT: RTRs with a wild type NOD2 gene; H/h: heterozygote RTRs with a mutated NOD2 allele in either SNP8, SNP12 or SNP13 region (since no heterozygous composites or homozygous mutants were detected); DM: double mutated patients or homozygous mutants; AE: atherosclerotic events.

## FIGURE LEGEND

**Figure 1.** A representative analysis of NOD2 gene polymorphism by restriction fragment length polymorphism (RFLP)-PCR. Agarose gel electrophoresis of RFLP-PCR after MspI, HhaI, or ApaI overnight restriction digest, respectively, for SNP8 (left panel) SNP12 (middle panel) and SNP13 (right panel) polymorphisms. **WT** corresponds to DNA from a wild type patient, while **h** corresponds to DNA from a heterozygote patient. **PCR** corresponds to undigested PCR products of 185 bp, 163 bp, and 151 bp, respectively, for SNP8, SNP12 and SNP13 polymorphisms. **MW** represents molecular weight marker where the 3 bands are 100bp, 200 bp and 300 bp in length. In **h** samples,  $\diamond$  symbol depicts the mutated allele, whereas  $\blacklozenge$  depicts the wild type allele.

**Figure 1**

