Genetic Variants of the NLRP3 Inflammasome Are Associated with Stroke in Patients with Rheumatoid Arthritis

Alf Kastbom, Lisbeth Ärlestig, and Solbritt Rantapää-Dahlqvist

ABSTRACT. ***Objective.*** Inflammasomes are intracellular protein complexes important for the production of pro-inflammatory cytokines. Studies have suggested that the NLRP3 inflammasome influences both the severity of rheumatoid arthritis (RA) and development of atherosclerosis. Therefore, we investigated whether functional genetic variants related to the NLRP3 inflammasome influence the risk of cardiovascular (CV) disease (CVD) in patients with RA.

Methods. The incidence of CVD was assessed in 522 patients with established RA by a retrospective survey of medical records in combination with a 6-year prospective followup. NLRP3-Q705K and CARD8-C10X genotypes were analyzed in relation to CVD by logistic regression, adjusting for traditional risk factors, antirheumatic treatment, and age at the onset of RA.

Results. Carriage of the NLRP3-Q705K minor allele was associated with an increased risk of stroke/transient ischemic attack (TIA; OR 2.01, 95% CI 1.0–4.1, p = 0.05), while CARD8-C10X was not associated with any type of CV event. Patients with ≥ 1 variant allele in both polymorphisms had an increased risk of CVD when compared with patients without variant alleles present in both polymorphisms (adjusted OR 3.05, 95% CI 1.42–6.54, p = 0.004). Stratification showed that this risk was confined to stroke/TIA (adjusted OR 5.09, 95% CI 2.27–11.44, p < 0.0001) and not to myocardial infarction (MI)/angina pectoris (adjusted OR 1.58, 95% CI 0.67–3.73). Risk estimates were consistently higher among female patients.

Conclusion. Genetic variants of the NLRP3 inflammasome influence the risk of stroke/TIA, but not of MI/angina pectoris in Swedish patients with established RA. (First Release July 15 2015; J Rheumatol 2015;42:1740–5; doi:10.3899/jrheum.141529)

Key Indexing Terms:
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Cardiovascular (CV) disease (CVD) is significantly overrepresented among patients with rheumatoid arthritis (RA). It is a major cause of the increased risk of premature death consistently associated with established RA[1,2]. Apart from traditional proatherosclerotic risk factors, inflammatory activity enhances the risk of CV events[3,4] and may possibly be modified by antirheumatic therapy[5,6].

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It is well established that local inflammation in vessel walls is crucial in the development of atherosclerotic lesions, and in patients without a concurrent inflammatory disease such as RA[7]. However, understanding of the mechanisms by which systemic inflammation increases the risk of clinical CVD in patients with RA remains incomplete. We have previously suggested a role for the interleukin 1β (IL-1β)-regulating NLRP3 inflammasome in the disease course of early RA because functional single-nucleotide polymorphisms (SNP) in the genes encoding NLRP3 and CARD8 were found to be associated with inflammatory activity and the subsequent need for therapy with tumor necrosis factor inhibitors (TNFi)[8,9]. Intriguingly, there is also increasing evidence for a role of the NLRP3 inflammasome in the development of atherosclerotic lesions. First, it was shown that the NLRP3 inflammasome was activated by cholesterol crystals, resulting in the secretion of IL-1β[10,11], and that this mechanism significantly contributed to atherogenesis in a mouse model[11]. Second, activation of the NLRP3 inflammasome was shown to accelerate macrophage lipid deposition and foam cell development[12].

Mutations in the gene encoding NLRP3 are associated with rare autoinflammatory diseases [e.g., cryopyrin-associ-

ated periodic syndromes (CAPS)] and dysregulated IL-1β production[13]. The Q705K variant (rs35829419), however, is a polymorphism with about 10% of the Swedish population being carriers of a minor allele[8,9,14]. NLRP3-Q705K causes significantly increased inflammasome-mediated release of IL-1β *in vitro*, although to a more moderate degree compared with the mutations causing CAPS[15]. CARD8 is an adaptor protein that both interacts with inflammasome components and inhibits nuclear factor-κB (NF-κB) transcription[16,17]. The CARD8-C10X polymorphism (rs2043211), which introduces an early stop codon, has been shown to impair the NF-κB–inhibiting properties of the protein[17], but functional studies in relation to inflammasomes are lacking. A number of reports have described interacting effects between NLRP3-Q705K and CARD8-C10X, for example, regarding RA[8], aortic abdominal aneurysms[18], inflammatory bowel disease[19,20], and plasma levels of IL-1β in healthy individuals[14].

Given that CVD is overrepresented among patients with RA, and that the NLRP3 inflammasome has been linked to both RA severity and the development of atherosclerosis, we hypothesized that the NLRP3-Q705K and CARD8-C10X polymorphisms increase the risk of CVD in patients with RA.

MATERIALS AND METHODS

Subjects. The study subjects were 560 patients with established RA from northern Sweden fulfilling the 1987 American College of Rheumatology RA classification criteria[21], consecutively recruited into the study during the first 4 months of 1995, 2000, and 2001[22]. Of these, 522 patients (382 women and 140 men) donated blood samples for DNA analysis. A thorough survey of all the available patient records from the onset of RA until inclusion in the study was performed retrospectively according to a structured study registration form. All patients were followed prospectively for 6 years from the inclusion date. CV events after the onset of RA were registered retrospectively according to the following definitions: (1) a myocardial infarction (MI) diagnosed by a clinical physiologist or cardiologist based upon typical changes in electrocardiograms according to the Minnesota code[23], together with the typical enzymatic pattern and/or echocardiographic verification; (2) an angina pectoris treated with coronary artery bypass graft surgery or percutaneous coronary intervention; (3) a stroke when an intracerebral hemorrhage or infarction had been diagnosed following computerized tomography or magnetic resonance imaging, or when a typical clinical picture with neurological deficits had persisted for more than 24 h; and (4) a transient ischemic attack (TIA) in those cases in which a focal neurological deficit of presumed ischemic origin had persisted for < 24 h. Hypertension (HTN) was defined by treatment for it. Total accumulated disease activity was analyzed from retrospective data by calculation of the number of tender and swollen joints, erythrocyte sedimentation rate (mm/h), and the physician's global assessment of disease activity as described by Baecklund, *et al*[24]. Treatment (≥ 6 mos) with oral corticosteroids, disease-modifying antirheumatic drugs, TNFi, and statins were recorded. The 6-year followup information was derived from the register of the National Board of Health in Sweden to obtain data on hospital inpatient care and/or death with a diagnosis of a new event, such as MI [defined by the 10th revision of the International Statistical Classification of Diseases and Related Health Problems (ICD-10) codes I21.0–9, I22.0–1, I22.8–9], angina pectoris with intervention (ICD-10 codes I20.0–I20.8–9 + Z 951 or 955 or FNG V9250, 9251, 9253), and/or stroke/TIA (ICD-10 codes I61.0–6, I61.8–9, I62.9, I63.0–6, I63.8–9, I64). These outcomes have been validated in a previous study with 96% concordance between hospital records and the registers[25].

The study protocol was approved by the Research Ethics Committee in Umeå, Sweden. All patients gave informed consent to participate in the study that was performed according to the principles of the Helsinki Declaration.

Laboratory analyses. Genomic DNA was extracted from buffy-coat cells using standard methods. HLA-DRB1 genotyping was performed using PCR sequence-specific primers from DR low-resolution kit and DRB1*04 subtyping kit (Olerup SSP AB). Shared epitope was defined as DRB1*0401/0404/0405/0408, as previously described in detail[26].

NLRP3-Q705K and CARD8-C10X were genotyped using commercially available TaqMan assays (Applied Biosystems) according to the manufacturer's instructions. The genotyping success rate was 99% for the CARD8-C10X polymorphism and 100% for NLRP3-Q705K. IgG-class anti-citrullinated protein antibodies were analyzed by ELISA with second-generation cyclic citrullinated peptides as antigen (Axis Shield Diagnostics; cutoff 5 U/ml).

Statistical methods. The chi-squared test or Fisher's exact test, when appropriate, was used for testing categorical data between groups, and logistic regression analyses were used to estimate OR for predicting variables for the dependent variable. For analysis of continuous data, the independent Student t test was used. All p values are 2-sided, and p values ≤ 0.05 were considered statistically significant. Calculations were performed using SPSS 22 software. Adjustments in the multiple logistic analyses were based on those factors that were significantly associated in simple logistic regression analyses. Empirical p values were calculated with Haploview 4.2, using 10,000 permutations.

RESULTS

Genotype frequencies were in Hardy-Weinberg equilibrium for both SNP ($p > 0.1$), and comparable with previous findings from Swedish datasets[8,9,14]. Table 1 shows demographic and clinical characteristics of the patients. From the onset of RA to the 6-year followup, 121 patients were recorded as undergoing a CV event(s): 74 had an MI, 20 had angina pectoris with intervention, and 50 experienced a stroke/TIA. Patients with registered CV event(s) after the onset of RA were older, had a longer duration of the disease, and had more often survived a CV event before the onset of RA as compared with those without a CV event(s). They were more likely to be men, more often had diabetes and/or HTN, and were more frequently treated with oral corticosteroids and statins. Therapy with TNFi was less common among those with a history of a CV event (Table 1).

The distribution CARD8-C10X genotypes did not differ significantly between patients with and without a CV event(s). However, the variant allele of NLRP3-Q705K was significantly more common among patients with a stroke/TIA event after the onset of RA (OR 2.01, 95% CI 1.0–4.06, p = 0.05, empirical p value = 0.086). This association was more pronounced among women (OR 3.50, 95% CI 1.54–7.95, p = 0.003, empirical p value = 0.013). Simple logistic regression analyses showed significant association between CV event and age at onset of RA, event preceding RA, HTN, and diabetes, and treatment with TNFi, statins, and corticosteroids. Adjusting for these factors in multiple logistic regression yielded similar results (all patients: OR 2.02, 95% CI 0.96–4.21, p = 0.06; women: OR 4.20, 95% CI 1.72–10.22, p = 0.002).

The combined CARD8 and NLRP3 genotypes were

Table 1. Clinical and demographic data for the patients with RA stratified by CV event after the onset of RA. Values are mean ± SD or n (%).

Characteristics	All Patients, n = 522	Patients without CV Event, n = 401	Patients with CV Event†, n = 121
Age, yrs	61.4 ± 13.1	60.7 ± 13.6	63.5 ± 11.3*
Age at onset, yrs	46.1 ± 15.1	44.2 ± 14.6	52.6 ± 14.8***
Disease duration, yrs	15.7 ± 12.2	15.0 ± 11.9	17.7 ± 13.1*
Women	382 (73.2)	306 (76.3)**	76 (62.8)
RF	476/520 (91.5)	364/399 (91.2)	112/121 (92.6)
ACPA	408/492 (82.9)	318/386 (82.4)	90/106 (84.9)
HLA-SE	335/502 (66.7)	259/390 (66.4)	76/112 (67.9)
Erosions	434/511 (84.9)	335/395 (84.8)	99/116 (85.3)
Accumulated disease activity††	4.6 ± 0.97	4.6 ± 1.0	4.7 ± 0.92
Ever smoked	250/517 (48.4)	189/400 (47.3)	61/117 (52.1)
Previous CV event‡	17/522 (3.3)	8/401 (2.0)	9/121 (7.4)**
Hypertension	179/522 (34.3)	117/401 (29.2)	62/121 (51.2)***
Diabetes	44/521 (7.3)	21/400 (5.0)	23/121 (14.9)***
DMARD, ever	474/522 (90.8)	363/401 (90.5)	111/121 (91.7)
Methotrexate, ever	280/521 (53.7)	222/401 (55.4)	58/120 (48.3)
Steroids, ever	298/520 (57.3)	213/399 (53.4)	85/121 (70.2)**
Lipid-lowering drugs, statins	50/522 (9.6)	26/401 (6.5)	24/121 (19.8)***
TNFi	84/522 (16.1)	78/401 (19.5)	6/121 (5.0)***

* $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$. † CV event defined as myocardial infarction, angina pectoris with intervention, stroke/transient ischemic attack. ††Calculated according to Baecklund, *et al*[24]. ‡ CV event before onset of RA. RA: rheumatoid arthritis; CV: cardiovascular; RF: rheumatoid factor; ACPA: anticitrullinated protein antibodies; HLA-SE: HLA-DRB1/shared epitope; DMARD: disease-modifying antirheumatic drug; TNFi: tumor necrosis factor inhibitor.

dichotomized according to the presence or absence of the variant allele in both SNP, i.e., patients carrying minor allele(s) in both SNP were compared with those patients who did not. In this model, logistic regression analysis showed that the combined genotype distribution (Table 2) differed significantly between those with and without any CV event (all: OR 2.33, 95% CI 1.22–4.45, p = 0.011, and women: OR 2.78, 95% CI 1.29–6.02, p = 0.009), in particular regarding stroke/TIA (all: OR 4.44, 95% CI 2.11–9.35, p < 0.0001, and women: OR 7.37, 95% CI 3.08–17.64, p < 0.0001; Table 2), but not for those with MI/angina pectoris (all and women: p = NS; Table 2). The significant results became even stronger in the adjusted multiple logistic regression analyses (Table 2), with OR consistently higher among women.

A logistic regression testing the interaction between NLRP3-Q705K and CARD8-C10X regarding CV event resulted in an OR 4.68 (95% CI 1.28–17.09). However, no significant interaction was detected for stroke/TIA or MI/AP, respectively.

DISCUSSION

Our study presents the novel finding that genetic variants of the NLRP3 inflammasome influence the risk of CVD in patients with RA, even after adjusting for traditional risk factors and RA treatment. This finding is supported biologically by the fact that atherogenic lipids are able to activate the NLRP3 inflammasome, and that this occurs in atherosclerotic plaques[11,12].

We chose to relate the combined NLRP3-Q705K and CARD8-C10X genotypes to CV risk, because synergistic effects between these SNP have been described repeatedly[8,14,18,20], albeit not exclusively[9]. Indeed, we found a significant gene-gene interaction in relation to CVD risk.

Subgroup analyses showed that the increased risk of a CV event was mainly confined to stroke/TIA and not to coronary artery disease (CAD). We have no obvious explanation for this difference because both outcomes are regarded as clinical manifestations of atherosclerosis. Stroke/TIA and CAD have, however, a number of disparities that may be relevant. For instance, while the rupture of lipid-loaded plaques is a common feature of coronary and carotid lesions, the clinical events caused by superficial erosions of proteoglycan-rich plaques with minor lipid cores and fewer inflammatory cell infiltrates mainly occur in CAD, but not in stroke/TIA[27,28]. In 1 study, this mechanism was ascribed to > 40% of the cases with sudden coronary death, and could hypothetically be less related to inflammasome functions, given the minor macrophage infiltration and lipid deposition in the lesion[28]. Further, leukocytes need a "priming signal" before the NLRP3 inflammasome can become activated, for example, through the ligation of Toll-like receptor 4 (TLR4), which was actually found to be expressed differently in diverse arteries[29]. Thus, although TLR4 expression was found to be upregulated in unstable carotid plaques as well as coronary arteries[30,31], local differences could hypothetically exist regarding prerequisites for inflammasome activation. Another

Table 2. The distribution of the compound NLRP3-Q705K/CARD8-C10X polymorphism and CVD in patients with RA. Data are comparing those without the combination of the variant allele in both SNP (Reference) with those having ≥ 1 variant allele in both SNP. Values are n (%) unless otherwise specified.

Table 2A. CV event.

Variables	CV Event		Unadjusted		Adjusted*	
	Without	With	p	OR (95% CI)	p	OR (95% CI)
All patients	CARD8-CC	CARD8-CX/XX				
NLRP3-QQ	370 (93.4)	104 (86.0)		Reference		Reference
NLRP3-QK/KK	26 (6.6)	17 (14.0)	0.011	2.33 (1.22–4.45)	0.004	3.05 (1.42–6.54)
Men						
NLRP3-QQ	88 (92.6)	40 (88.9)		Reference		Reference
NLRP3-QK/KK	7 (7.4)	5 (11.1)	NS	1.57 (0.47–5.25)	NS	2.14 (0.50–9.07)
Women						
NLRP3-QQ	282 (93.7)	64 (84.2)		Reference		Reference
NLRP3-QK/KK	19 (6.3)	12 (15.8)	0.009	2.78 (1.29–6.02)	0.008	3.37 (1.37–8.31)

Table 2B. Stroke/TIA.

Variables	Stroke/TIA		Unadjusted		Adjusted*	
	Without	With	p	OR (95% CI)	p	OR (95% CI)
All patients	CARD8-CC	CARD8-CX/XX				
NLRP3-QQ	436 (93.4)	38 (76.0)		Reference		Reference
NLRP3-QK/KK	31 (6.6)	12 (24.0)	< 0.0001	4.44 (2.11–9.35)	< 0.0001	5.09 (2.27–11.44)
Men						
NLRP3-QQ	111 (91.7)	17 (89.5)		Reference		Reference
NLRP3-QK/KK	10 (8.3)	2 (10.5)	NS	1.31 (0.26–6.48)	NS	1.24 (0.20–7.58)
Women						
NLRP3-QQ	325 (93.9)	21 (67.7)		Reference		Reference
NLRP3-QK/KK	21 (6.1)	10 (32.3)	< 0.0001	7.37 (3.08–17.64)	< 0.0001	8.83 (3.34–23.35)

Table 2C. MI event.

Variables	MI Event		Unadjusted		Adjusted*	
	Without	With	p	OR (95% CI)	p	OR (95% CI)
All patients	CARD8-CC	CARD8-CX/XX				
NLRP3-QQ	397 (92.3)	77 (88.5)		Reference		Reference
NLRP3-QK/KK	33 (7.7)	10 (11.5)	NS	1.56 (0.74–3.30)	NS	1.58 (0.67–3.73)
Men						
NLRP3-QQ	98 (92.5)	30 (88.2)		Reference		Reference
NLRP3-QK/KK	8 (7.5)	4 (11.8)	NS	1.63 (0.46–5.80)	NS	2.02 (0.47–8.64)
Women						
NLRP3-QQ	299 (92.3)	47 (88.7)		Reference		Reference
NLRP3-QK/KK	25 (7.7)	6 (11.3)	NS	1.53 (0.60–3.92)	NS	1.22 (0.39–3.81)

* Adjusted with age at onset of RA, event preceding RA, hypertension, and diabetes, and treatment with TNFi, statins, and corticosteroids. CVD: cardiovascular disease; RA: rheumatoid arthritis; SNP: single-nucleotide polymorphism; CV: cardiovascular; TIA: transient ischemic attack; MI: myocardial infarction; TNFi: tumor necrosis factor inhibitor; NS: not significant.

important "first hit" in inflammasome activation is the binding of adenosine triphosphate to the purinergic $P2X_7$ receptor, and functional SNP of the *$P2X_7$* gene have been associated with the risk of stroke[32]. This should encourage future studies to include the genes of several inflammasome-related signaling pathways.

Most studies into the risk of CVD present results adjusted for sex, but we chose to present sex-specific OR because a report suggested a sex-specific effect of NLRP3-Q705K on the risk of an MI, in which the minor allele was associated with a decreased risk in females, but not in males[33]. In contrast to the previous study, we found the minor allele to be independently associated with an increased risk of any CV event, but not specifically to CAD. The difference in study

participants, i.e., a population-based approach versus established patients with RA, probably influenced this disparity. As possible sex differences regarding inflammasome functions await clarification, we believe that the observed differences between the sexes should be interpreted with caution.

A previous large study on Spanish patients with RA found no relationship between CARD8-C10X and the risk of an MI, stroke, or intima media thickness[34]. Likewise, a Swedish population-based investigation found no association between CARD8 genotype and the risk of an MI[35]. Consistent with these reports, we found no independent effect of CARD8-C10X on any CV event. In contrast, a recent Chinese population-based investigation found that the minor allele of CARD8-C10X was associated with a reduced risk of stroke, although NLRP3 genetic variants were not accounted for[36]. We found that the presence of the minor allele potentiated the effect of NLRP3-Q705K in terms of stroke, increasing the OR from 2.0 to 4.4. Recently, Ito, *et al* showed that the CARD8 protein is a negative regulator of the NLRP3 inflammasome, but that the CARD8 protein cannot interact with NLRP3 when CAPS-associated mutations are present[16]. We hypothesize that the much more common NLRP3-Q705K polymorphism allows CARD8-NLRP3 interaction[15]. Further, whereas CARD8-C10X does not appear to influence IL-1β from NLRP3-wildtype cells[16], C10X may be of importance if the NLRP3-Q705K minor allele is present. However, this hypothesis remains to be tested. Of note, the previous functional study of NLRP3-Q705K was performed on CARD8-C10X heterozygous cells[15].

The NLRP3 inflammasome has shown to be important in mediating ischemic injury in experimental models of stroke. For instance, NLRP3-deficient mice were shown to have smaller infarct size[37], and modulation of NLRP3 and/or caspase-1 activity may improve outcome after cerebral ischemia[38,39]. Unfortunately, because of the lack of radiological and clinical followup data, we are unable to investigate a possible effect of the NLRP3-Q705K and CARD8-C10X on stroke severity.

We present evidence that NLRP3 inflammasome genetic variants influence the risk of stroke/TIA, but not MI/angina pectoris among Swedish patients with established RA. Future studies should include genetic variants in upstream signaling pathways. Also, the possible clinical value of NLRP3 and CARD8 genotyping of patients with RA in relation to CVD risk and prevention needs further attention in larger patient cohorts.

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