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The *NFKB1* ATTG ins/del Polymorphism and Risk of Coronary Heart Disease in Three Independent Populations

Ulla Vogel[1,2], **Majken K. Jensen**[3], **Karen Margrete Due**[4], **Eric B. Rimm**[3,5,6], **Håkan Wallin**[1], **Michael R.S. Nielsen**[4], **Anne-Pernille T. Pedersen**[4], **Anne Tjønneland**[7], and **Kim Overvad**[4,8]

[1]National Research Centre for the Working Environment, Copenhagen, Denmark

[2]National Food Institute, Technical University of Denmark, Soborg, Denmark; Institute of Science, Systems and Models, Roskilde University, Roskilde, Denmark

[3]Department of Nutrition, Harvard School of Public Health, Boston, MA, USA

[4]Department of Cardiology, Center for Cardiovascular Research, Aalborg Hospital, Aarhus University Hospital, Denmark

[5]Department of Epidemiology, Harvard School of Public Health, Boston, MA, USA

[6]Department of Medicine, Channing Laboratory, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA

[7]Danish Cancer Society, Copenhagen, Denmark

[8]Department of Epidemiology, School of Public Health, Aarhus University, Aarhus, Denmark

Abstract

Aim—Inflammation is a risk factor for coronary heart disease (CHD). A common deletion-allele in the promoter region of *NFKB1* results in lower protein levels of the NF-κB p50 subunit. Recent evidence suggests that the NF-κB p50 dimer has anti-inflammatory effects. We aimed to investigate the association of the functional ATTG *NFKB1* insertion/deletion variant with risk of CHD in three independent prospective studies of generally healthy men and women.

Methods and Results—The *NFKB1* ins/del polymorphism was genotyped in studies of CHD nested within the Diet, Cancer and Health (DCH) study, the Health Professionals Follow-up (HPFS) and the Nurses' Health (NHS) studies, totaling 1008, 428 and 439 cases, respectively. The minor allele frequency in the combined sample was 0.38 among controls. In a pooled analysis, the relative risk (RR) among heterozygous men and women was 1.22 (95% CI: 1.07–1.40), compared to the most common ins/ins genotype. The RR among homozygotes was 1.20 (95% CI: 0.94–1.53). There was no evidence of an allele-dosage effect, and in a dominant model the RR among del-allele carriers was 1.22 (95% CI: 1.07–1.39). The risk was similar in women and men (RR was 1.20 in women and 1.23 in men, respectively). The *NFKB1* variant was not associated with plasma lipid levels, but del-carriers had lower levels of C-reactive protein.

Conclusions—The *NFKB1* promoter variant, previously shown to cause partial depletion of NF-kB p50, was associated with a higher risk of CHD in three independent prospective studies of generally healthy Caucasians.

Address for Correspondence: Ulla Vogel, National Research Centre for the Working Environment, Lersø Parkallé, DK-2100, Copenhagen Ø, Denmark, ubv@nrcwe.dk, fax: +45 3916 5201, phone: +45 3916 5227.

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Keywords

NFkappaB; inflammation; polymorphism; population-based

Introduction

Coronary heart disease (CHD) is a major cause of morbidity and mortality in the western world. The transcription factor NF-κB is a key regulator of many cellular processes and genes involved in modulation of inflammation. Thus, many genes that are relevant to the pathogenesis of atherosclerosis are regulated by NF-κB (1). The p50 subunit of NF-κB encoded by *NFKB1* seem to be specifically involved in anti-inflammatory effects (2). Thus, the p50 homodimer represses transcription of pro-inflammatory cytokines like TNF and IL12 and stimulates transcription of the anti-inflammatory cytokine IL10 (2; 3).

The *NFKB1* gene encodes both the subunits p105 and p50 of the transcription factor NF-κB by alternative splicing (4). A functional ATTG insertion/deletion (ins/del) polymorphism in the promoter region of *NFKB1* gene destroys a transcription factor binding site, resulting in differential expression (5; 6). The del-allele results in lower transcript levels and protein levels of both p50 and p105. Consequently, carriers of the del-allele have lower levels of functional NF-κB p50 (5).

Thus, it may be that del-carriers are genetically determined towards a higher inflammatory response.

Our aim was to examine the associations of the *NFKB1* promoter ATTG ins/del polymorphism with CHD risk in three independent studies of generally healthy Caucasians and the potential association with blood lipids and C-reactive protein (CRP).

We therefore hypothesized that the *NFKB1* polymorphism would be associated with risk of CHD and that del-allele carriers, with a defective anti-inflammatory p50 NF-κB signalling pathway, would be at higher risk of CHD than homozygous carriers of the ins-allele.

Methods

Study populations

The Diet, Cancer, and Health (DCH) study was initiated in 1993–1997 when 57,053 Danish born residents, aged 50 to 64 years and free of cancer, participated in a clinical examination and detailed lifestyle survey (7). Blood was sampled at baseline in the study clinic and stored as plasma, serum, lymphocytes, and erythrocytes at −150 °C. The Nurses' Health Study (NHS) enrolled 121,701 female nurses aged 30 to 55 who returned a mailed questionnaire in 1976 regarding lifestyle and medical history. The Health Professionals Follow-up Study (HPFS) enrolled 51,529 males aged 40 to 75 who returned a similar questionnaire in 1986. Participants of both cohorts have received follow-up questionnaires biennially to record newly diagnosed illnesses and to update lifestyle and dietary information.

Detailed descriptions of the study cohorts have been published previously (8–10). In the US cohorts, a blood sample was requested from all active participants in 1989–1990 in NHS and 1993–1995 in the HPFS. A total of 32,826 female participants in NHS and 18,224 men in the HPFS returned samples. Nested case-control studies were designed using incident CHD, with non-fatal myocardial infarction (MI) and fatal CHD as the outcome. Cases were identified primarily through review of medical records, as previously described (11). Among participants who provided blood samples and who were without cardiovascular disease or

cancer at blood draw, we identified 474 women among 32,826 study participants who sustained an incident MI/CHD between blood draw and follow up, and 454 men among 18,224 study participants in the HPFS. Using risk-set sampling (12), controls were selected randomly and matched in a 2:1 ratio on age, smoking, and month of blood return.

In the DCH, a case-cohort study was designed using incident validated cases of acute coronary syndrome (including unstable angina, non-fatal and fatal MI) as the outcome (8; 13–16). Information on the disease endpoint was obtained by linking the participants (via the unique identification number assigned to all Danish citizens) with central Danish registries of hospital discharge diagnoses and causes of death (ICD-8 codes 410–410.99, 427.27 and ICD-10 codes I20.0, I21.x, I46.x). In total, 1150 incident cases were verified between baseline and Jan 1st, 2004, the date of the last available update from the hospital discharge register among 57, 053 participants. Consistent with the case-cohort design, 1800 participants were selected from the entire DCH study at random (sub-cohort members will also be referred to as controls throughout the paper although this group includes 34 participants who later became cases).

Laboratory analysis methods

Plasma lipids, lipoproteins, and apolipoproteins were assessed using standard methods with reagents from Roche Diagnostics (Indianapolis, IN) and Bayer Diagnostics (New York, NY). In the DCH study C-reactive protein (CRP) was not previously assessed, but in the NHS and HPFS, plasma levels of CRP were measured as described (17).

Genotypes of *NFKB1* ATTG ins/del (rs28362491) were determined by Taqman allelic discrimination (ABI 7500/7900HT, Applied Biosystems) as described (18). The case status of the samples was blinded during genotyping. Controls were included in each run and repeated genotyping of a random 10% subset yielded 100% identical genotypes. The call rates were 99.9% for the DCH cohort, 96.3 % for NHS, and 95.5% for HPFS.

Statistical analysis

Conformity with Hardy-Weinberg equilibrium was tested using a two-sided Chi-test among the controls. Relative rate ratios (RRs) and 95% confidence intervals (CIs) for the association between genotype and CHD were estimated using Cox proportional hazard regression with Kalbfleisch and Lawless weights and robust variance suitable for the DCH case-cohort data, and conditional logistic regression for the NHS and HPFS nested case-control data (19). We calculated sex-specific estimates for the association between the *NFKB1* polymorphism and risk of CHD in the DCH cohort to allow for direct comparisons with the all female NHS and all male HPFS cohorts. All analyses included adjustment for age, smoking, alcohol, BMI, hypertension, hypercholesterolemia, diabetes and use of non-steroid anti-inflammatory drugs (NSAID) including aspirin. We tested for statistical interaction between the *NFKB1* polymorphism and cardiovascular risk factors (smoking status, alcohol intake, BMI and NSAID by using a likelihood ratio test in models with and without their joint effects. In total, numbers with information available on genotype, and covariates were: 2663 participants including 1008 cases in DCH subdivided into 1644 men (769 male cases and 26 who were both cases and sub-cohort members) and 1019 women (239 cases and 7 who were both cases and sub-cohort members); 439 cases, 845 controls in NHS; 428 cases, 875 controls in HPFS.

To pool the estimates from the three two study populations, we calculated the weighted average of the log RRs with weights according to the study-specific standard errors using the DerSimonian and Laird random-effects model. To investigate whether the association for the *NFKB1* ins/del polymorphism with CHD was different for men and women, we

analyzed the DCH women and men separately and next pooled the estimate for women with the NHS data and the estimate for men with the HPFS estimate. In the final model, all sex- and study specific RRs were pooled.

We estimated mean lipid levels according to genotype in the randomly selected DCH comparison group and the NHS and HPFS control groups using least square linear regression adjusted for the above-mentioned covariates. In the NHS and HPFS mean CRP levels across genotypes were estimated in a similar fashion (CRP was not measured in the DCH cohort).

Analyses were performed using SAS 9 (SAS Institute Inc., Cary, NC) and STATA 10 (STATA Corp., College Station, TX).

Results

Three independent prospective cohort studies were included. The Danish Diet, Cancer and Health (DCH) cohort included both men and women, whereas the US-based Nurses' Health Study (NHS) and Health Professionals Follow-up Study (HPFS) were gender-specific. Table 1 shows baseline characteristics of cases and controls in the three study populations. The Danish participants on average drank more alcohol and smoked more than the US cohort members. The study populations had similar body weight but differed in the baseline prevalence of diabetes and hypercholesterolemia, which was more often diagnosed and reported in the US cohorts.

The *NFKB1* genotype distribution was in Hardy-Weinberg equilibrium in the control study populations (p-values for deviation from HWE were 0.77, 0.98, 0.23 and 0.10, respectively, for DHC men, DHC women, HPFS, and NHS). The allele frequency of the variant del-allele was similar in the control groups from the three cohorts. In the combined samples, the minor allele frequency among controls was 0.38.

All three studies showed evidence that the del-allele was associated with a higher risk of CHD (Table 2). We did not see evidence of between-study heterogeneity, and thus used meta-analysis to pool the relative risks (RRs) estimated in each study and gender. The risk among heterozygotes was 1.22 (95% CI: 1.07–1.40), compared to the most common ins/ins genotype. The relative risk (RR) among homozygous del-allele carriers was 1.20 (95% CI: 0.94–1.53). The results did not suggest a gene-dosage effect, but there was no statistical evidence against it. In a dominant model the RR among del-allele carriers was 1.22 (95% CI: 1.07–1.39).

Although there was some variation in the estimated associations between the individual substudies, the wide confidence intervals for the study-specific estimates indicate low statistical power for reliable assessment in either study by itself. Pooling the gender and study-specific estimates, however, increases the statistical power while also allowing for optimal statistical analysis in each dataset taking into account study design and gender-specific covariates. The pooled estimates for women and men were very similar (the RR for del-allele carriers was 1.20 [95% CI: 0.97–1.48] in women and 1.23 [95% CI: 1.05–1.46] in men, respectively).

In separate evaluations of interactions between the *NFKB1* ins/del polymorphism and smoking status, alcohol intake, NSAID use, or BMI in relation to risk of CHD in the DCH cohort, we did not find any evidence for statistically significant interactions as only additive effects were seen (data not shown). There was no association between *NFKB1* ins/del and hypertension in the DCH cohort. There was no interaction between treatment for hypercholesterolemia and the *NFKB1* ins/del polymorphism among men, neither in HPFS

(p=0.70) nor in DCH (0.90). Among women, indications of interaction was found in DCH (p=0.04) but not in NHS (p= 87). As there was no interaction in the three other study groups, and there were only 10 CHD cases and 3 non-cases who received treatment for hypercholesterolemia among the women in the DCH cohort, the interaction is likely to be a chance finding.

We evaluated the association between the *NFKB1* ins/del polymorphism and plasma biomarkers in the generally healthy comparison groups. Except for observed higher LDL-C concentration among NHS participants who were homozygous del-allele carriers, no associations between the *NFKB1* ins/del polymorphism and blood lipid levels were observed. Plasma levels of CRP have been measured in the HPFS and NHS (17). Del-allele carriers had lower plasma levels of CRP in both studies, although the association between genotype and plasma levels of CRP was only statistically significant in the NHS (p=0.02) (Table 3).

Discussion

The present findings suggest that carriers of the del-allele of *NFKB1* ATTG ins/del are at higher risk of CHD and have lower plasma levels of CRP.

NF-κB names a number of different transcription factors that are homo- or heterodimers of p65, p50, p105, C-rel and relB (2; 20). The target gene specificity of NF-κB is determined by the subunit type. NF-κB is involved in both inflammatory and anti-inflammatory processes in atherogenesis (21). The p50 subunit encoded by *NFKB1* has both pro- and anti-inflammatory properties. As part of the p65/p50 NF-κB transcription factor complex, it is pro-inflammatory, controlling transcription of pro-inflammatory cytokines such as TNF and IL1β (20). Conversely, p50 has anti-inflammatory properties in the p50 homodimer ($p50_2$), which represses transcription of pro-inflammatory cytokines like TNF and IL12 and stimulates transcription of the anti-inflammatory cytokine IL10 (2; 3; 22). The relative abundance of p50/p65 heterodimers and p50 homodimers will therefore determine the magnitude of inflammation by balancing the pro-inflammatory and anti-inflammatory response (2).

We hypothesize that partial depletion of p50 will disfavor the anti-inflammatory response because the formation of the pro-inflammatory p65/p50 heterodimer depends on the concentration of p50, while the formation of the anti-inflammatory p50 homodimer depends on concentration of p50 squared. Consequently, the anti-inflammatory response is more affected by the genetically determined partial p50 depletion of the del-allele. Our observation that del-allele carriers of *NFKB1* ins/del ATTG are at higher risk of CHD may therefore indicate that inflammation is a risk factor for CHD. The del-allele causes partial depletion of p50, resulting in preferential depletion of NF-κB p50 dimer-mediated anti-inflammatory effects such as repression of transcription of pro-inflammatory cytokines and production of the anti-inflammatory cytokine IL10.

We chose to study the ins/del *NFKB1* polymorphism because of the proven functionality and biological effects (5; 6). Deletion of one ATTG repeat in the promoter region of *NFKB1* abolishes the binding of a nuclear factor as shown in colon tissue and in cell lines and leads to less promoter transcriptional activity *in vitro* and less p50 biosynthesis (5). The NF-κB p50 dimer activates transcription of *CRP* together with IL-6 and IL-1β (23). We observed that del-allele carriers of *NFKB1* polymorphism had lower baseline plasma CRP levels in an allele-dose dependent manner. The association thus supports the suggested functionality of the studied polymorphism.

We observed no overall gene-dosage effect of the studied polymorphism in relation to CHD risk although the observed risk estimates and confidence intervals also were compatible with an additive gene-dosage model. A dosage effect was observed in one of the four gender- and study-specific strata for CHD, but it is possible that larger studies would be needed to substantiate a potentially small gene-dosage effect. Alternatively, it could reflect that p50 dimer-dependent transcription of anti-inflammatory genes also depends on the availability the co-factors IL1β and IL6. These two cytokines are transcribed by the p65/p50 dimer, and would therefore be less affected by the partial p50 depletion than p50 dimer formation. It is also possible that the balancing between an inflammatory and an anti-inflammatory response has a threshold effect in relation to the biological effect on CHD risk.

We found a positive correlation between the number of functional *NFKB1* ins-alleles and plasma CRP levels. Plasma CRP levels have been shown to be a risk factor for CHD in prospective studies, but in a recent large study, polymorphisms and haplotypes of *CRP* that were associated with high CRP levels did not predict an increased risk of CHD (24; 25). This suggests that the association between CRP plasma levels and CHD may be non-causal. This, in turn, could indicate that either the p50 dimer-dependent anti-inflammatory effects or other acute phase proteins which co-vary with CRP, such as SAA (26) could be causally related to CHD.

We conducted our analyses in three independent cohorts of Caucasian men and women who were free from cardiovascular disease at baseline. Our cases and controls were selected from the same cohorts, all with almost complete follow-up of the participants which should minimize the risk for selection bias. For all participants, information on lifestyle factors was collected at enrolment, which minimized the risk for differential misclassification between the cases and controls. The present study includes more than 1800 cases. The studied ins/del polymorphism is very frequent with a MAF of 0.38. The resulting narrow confidence intervals and the consistent results are thus unlikely to be chance findings. There was substantial variation in the prevalence of self-reported diagnosis of diabetes, hypertension and hypercholesterolemia across the studied cohorts. Most likely, this reflects the differences in US versus Europe with regard to diagnosis and medication use. Our analyses were therefore adjusted for hypertension, diabetes and hypercholesterolemia in addition to a number of lifestyle factors. Hypertension, diabetes and hypercholesterolemia are, however, most likely also intermediate variables between inflammation and CHD. They also reflect other potential confounders, which we did not take into account in the analyses. The adjusted analyses are thus less likely affected by confounding. The presence of an association in these analyses further indicates that other mechanisms than development of hypertension, diabetes, and hypercholesterolemia are responsible for the association between inflammation and CHD.

The studied insertion-deletion polymorphism has not been identified as a risk locus in recent comprehensive genome-wide association analysis of CHD (27) but, to our knowledge, the polymorphism is not included in the genome wide arrays. The polymorphism was reported to be in complete linkage with the single nucleotide polymorphism Exon1 +252C>G (rs72696119) in a study of 12 persons (5). However, this polymorphism is also not present on any genome-wide arrays or in the HapMap database.

In conclusion, our findings suggest that carriers of the del-allele of the functional *NFKB1* ATTG ins/del promoter polymorphism are at higher risk of CHD than homozygous ins-carriers. More research is warranted to disclose the biological mechanism that might explain this association.

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Table 1

Characteristics of cases and controls among men and women in the Diet, Cancer and Health study (DCH), among men in the Health Professionals Follow-Up Study (HPFS), and among women in the Nurses' Health Study (NHS).*

	DCH (both sexes)				HPFS (only men)		NHS (only women)	
	Men		Women		Men		Women	
Variable	**Cases (n=769)**	**Comparison† (n=870)**	**Cases (n=239)**	**Comparison† (n=773)**	**Cases (n=428)**	**Controls (n=875)**	**Cases (n=439)**	**Controls (n=845)**
Age (yrs)	58.0±4.5	56.6±4.4	59.0±4.1	56.4±4.3	64.2±8.7	64.3±8.6	59.9±6.4	59.9±6.4
BMI (kg/m^2)	27.5±3.7	26.6±3.5	26.7±4.7	25.4±4.4	26.1±3.2	25.6±3.4	26.5±5.3	25.1±4.3
Current smoker	59.0%	37.7%	59.4%	36.5%	9.8%	8.6%	26.7%	26.6%
Alcohol (g/d)	25.4±24.95	28.1±24.6	11.0±14.3	13.3±14.0	10.3±14.7	13.1±16.7	4.6 ±8.6	6.1±10.2
Postmenopausal	N/A	N/A	75.7%	75.3%	N/A	N/A	85.0%	83.1%
Diabetes§	5.2%	2.6%	5.0%	1.0%	9.6%	3.5%	15.3%	6.4%
Hypercholesterolemia‡	12.1%	9.6%	17.3%	6.0%	48.6%	40.6%	54.0%	40.7%
Hypertension§	22.3%	13.0%	39.6%	15.9%	37.2%	28.9 %	51.5%	27.8%
Lipid concentrations (mmol/L)								
Total cholesterol	6.31±1.04	5.98±0.96	6.60±1.30	6.10±1.04	5.45±1.00	5.24±0.94	6.04±1.02	5.88±1.05
Triglycerides	2.40±1.48	2.04±1.20	2.13±1.72	1.58±1.11	1.84±1.25	1.52±1.34	1.65±0.98	1.34±0.79
LDL-C	3.92±0.92	3.61±0.84	4.03±1.03	3.54±0.94	3.46±0.88	3.26±0.80	3.71±0.92	3.51±0.96
HDL-C	1.34±0.30	1.46±0.35	1.61±0.37	1.81±0.43	1.09±0.29	1.19±0.32	1.35±0.39	1.54±0.43

Values are means ± standard deviation of continuous covariates or percentages. In the NHS and HPFs, triglycerides were only measured in fasting participants at blood draw: HPFS: 65%, NHS: 79%. In DCH, all lipid measures were taken from non-fasting participants.

*In the NHS and HPFS, matching criteria were: age, smoking and date of blood sampling.

§Self-reported in questionnaire.

†Random sample of cohort at baseline (includes 33 cases)

‡Diagnosed with hypercholesterolemia or reporting to use cholesterol-lowering medication.

Table 2

Minor allele frequencies (MAF), rate ratio and 95% confidence intervals of CHD according to *NFKB1* ins/del polymorphism in the Diet, Cancer and Health (DCH) study, the Health Professionals Follow Up Study (HPFS) and Nurses' Health Study (NHS).

	MAF (s.e)		N (cases/controls)			Rate ratio (95% confidence interval)					
***NFKB1* genotype**	**Cases**	**Control**	**Ins/Ins**	**Ins/Del**	**Del/Del**	**Ins/Ins**	**Ins/Del**	**p**	**Del/Del**	**p**	**Del-carrier (dominant model)**	**p**
DCH men	0.41 (0.01)	0.37 (0.01)	268/352	368/395	133/123	1.0	1.23 (0.97–1.55)	0.09	1.54 (1.12–2.13)	0.008	1.30 (1.04-1.63)	0.02
DCH women	0.39 (0.02)	0.39 (0.01)	92/289	110/365	37/119	1.0	1.17 (0.80–1.73)	0.42	1.00 (0.581.72)	1.00	1.13 (0.78-1.62)	0.52
HPFS (men)	0.39 (0.02)	0.38 (0.02)	154/346	217/389	57/140	1.0	1.24 (0.96–1.61)	0.05	0.93 (0.64–1.35)	0.24	1.16 (0.91-1.48)	0.29
NHS (women)	0.40 (0.02)	0.38 (0.01)	164/336	200/370	75/139	1.0	1.22 (0.93–1.59)	0.58	1.29 (0.89–1.85)	0.39	1.23 (0.96-1.59)	0.11
Pooled men[†]	0.40	0.38	422/698	585/784	190/263	1.0	1.24 (1.04–1.47)	0.02	1.21 (0.73–1.99)	0.46	1.23 (1.05-1.46)	0.01
Pooled women[†]	0.39	0.39	256/625	310/735	112/258	1.0	1.20 (0.96–1.50)	0.11	1.19 (0.88–1.61)	0.26	1.20 (0.97-1.48)	0.09
Pooled men and women[†]	0.40	0.38	678/1323	895/1519	302/521	1.0	1.22 (1.07–1.40)	0.004	1.20 (0.94–1.53)	0.14	1.22 (1.07-1.39)	0.003

[*] Cox proportional hazard regression models in DCH (stratified by sex). Conditional logistic regression models were run in HPFS data (stratified by matching factors; age, smoking, blood draw). All models are adjusted for BMI, alcohol intake, NSAID use, hypertension, diabetes and hypercholesterolemia (age and smoking in DCH).

[†] Meta-analysis using random effects model.

Table 3

Least square mean C-reactive protein (CRP) plasma levels according to *NFKB1* genotypes in substudies of the Health Professionals Follow Up Study (HPFS) and Nurses' Health Study (NHS).

Plasma CRP levels (mg/L)				
	HSPS (men)		NHS (women)	
NFKB1	**Mean**	**p (compared to reference)**	**Mean**	**p (compared to reference)**
Ins/Ins	2.54	reference	3.28	Reference
Ins/Del	2.16	0.39	2.58	0.01
Del/Del	2.10	0.32	2.02	0.05
p*	0.54		0.02	

Least square means in the controls of the NHS and HPFS. Adjusted for age, BMI, alcohol, fasting status, smoking, family history of MI, and menopause status (women only).

p* test of linear association between genotype and CRP level.