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Gene variants in the NF-KB pathway (*NFKB1*, *NFKBIA*, *NFKBIZ*) and risk for early-onset coronary artery disease

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ABSTRACT

The nuclear-factor kappa-beta (NF-KB) is a driver of inflammation, and plays an important role in the pathogenesis of atherosclerosis and coronary artery disease (CAD). Early-onset CAD is defined as a coronary ischaemic episode at an age ≤55 years, and in our population was strongly associated with male sex and smoking. Our aim was to determine whether common variants in three NF-KB genes were associated with early-onset CAD. We studied 609 patients with early-onset CAD and 423 healthy controls, all male. Allele and genotype frequencies for the *NFKB1* rs28362491 (-94 delATTG) and *NFKBIA* rs8904 were not significantly different between the two groups. For the *NFKBIZ* rs3217713, the deletion allele was significantly more frequent in the patients than in controls (0.27 vs. 0.22; p = 0.004). Deletion-carriers were more frequent in the patients (p < 0.001), with an OR = 1.48 (95%CI = 1.15–1.90). We performed a multiple logistic regression (linear generalized model) with smoking, hypercholesterolemia, type 2 diabetes, hypertension, and the rs3217713 deletion carriers remained significantly associated with early-onset CAD (p = 0.01).

In our population, the *NFKBIZ* variant was an independent risk factor for developing early-onset CAD.

1. Introduction

The nuclear factor kappa-beta (NF-KB) is a transcription factor that regulates the expression of multiple genes that encode key components of immunological processes. The functional NF-KB transcription factor is a complex of at least one Rel protein (RelA, RelB, RelC) and the p50 or p52 proteins. As homo or heterodimers, p50/p52 act as repressors of NF-KB site transcription, but also participates in gene transactivation by forming heterodimers with the Rel peptides [1,2]. The NF-KB activity is regulated by several inhibitors in the cytoplasm that prevent its translocation to the nucleus [3]. The NF-KB is also regulated by nuclear proteins that regulate its binding to the DNA [4]. The deregulation of the components of the NF-KB pathway could thus result in immune-mediated and inflammatory pathological processes [5].

Coronary artery disease (CAD) is mainly caused by the development of atherosclerotic lesions in coronary vessels. Atherosclerosis and CAD are associated with several acquired/environmental risk factors, such as smoking, hypertension, hypercholesterolaemia, and diabetes. In addition, an inherited predisposition contributes to the development of atherosclerosis and CAD [6]. The genes that contribute to CAD-risk encode proteins involved in vascular endothelial physiology and atheroma development. Inflammation plays an important role in the development of atherosclerosis [7,8]. Because the NF-KB regulates many of the proinflammatory genes linked to atherosclerosis, the NF-KB pathway has been regarded as proatherogenic through its role in vascular inflammation, proliferation of vascular smooth muscle cells, or foam cell formation [9,10]. In contrast, the inhibition of NF-KB might protect from atherosclerosis development [11,12].

The association of nucleotide variants (polymorphisms) in the NF-KB genes with disease-risk has been widely investigated in several immune-mediated traits. One of the best characterised variants is rs28362491, a biallelic 4 nucleotides insertion/deletion (indel) in the

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NFKB1 promoter (-94 delATTG) that has been associated with several immuno-mediated and cancer and cardiovascular diseases [13–17]. This polymorphism has been linked to differences in *NFKB1* gene expression, with the deletion showing less promoter activity [13]. This functional effect could explain the reported disease-associations. Variants in the genes for other key components of the pathway, such as the NF-KB inhibitors, have also been associated with several diseases [15,18–23].

Few studies have addressed the role of NF-KB inhibitors gene variants in the risk of developing CAD. A promising variant was rs696 A/G, a SNP in the 3´ untranslated region of the *NFKBIA* gene. This nucleotide change would affect gene expression and activation of the NF-KB pathway through a mechanism that implicates the binding of a microRNA [19,24]. In vitro luciferase reporter assays showed that the G allele significantly decreased the stability of *NFKBIA* mRNA through the enhanced binding of miR-449a. Moreover, the NFKBIA protein levels in AA + AG tumor tissues were significantly higher than in those with the rs696 GG genotype [24]. These findings were in favor of a lesser amount of NF-KB inhibition in individuals with the A allele, that in turn could have an increased risk for inflammatory processes.

In addition to the classical cytoplasmic inhibitors (such as NFKBIA) the NF-KB activity is regulated by atypical proteins that are located in the nucleus. Among these is the IκBζ (*nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor zeta protein*) encoded by the *NFKBIZ* gene, that regulates the transcriptional activity of NF-KB [25]. IκBζ is induced by several pro-inflammatory molecules and has been identified as a new and important player in the pathogenesis of cancer and several immune-mediated processes [26–29]. Recently, we described a significant association between a common *NFKBIZ* polymorphism (rs3217713, a 20 nt intronic indel) and Psoriasis [30]. Although no functional link with gene expression was demonstrated, this was an intronic variant near the exon-intron boundary and could thus affect the RNA splicing.

Our aim was to determine the genetic epidemiology of common NF-KB gene polymorphisms in relation to the risk of developing CAD, and for this purpose we performed a case-control study comparing the frequency of *NFKB1*, *NFKBIA,* and *NFKBIZ* variants in patients with early-onset CAD and healthy population controls.

2. Methods

2.1. Study subjects

The Study involved a total of 609 CAD patients and 423 healthy population controls. Table 1 summarizes the main characteristics of these patients and controls. They were recruited for a case-control research to characterize the genetic variation that contributes to early-onset CAD in the population of Asturias (a Northern Spain region, total population 1 million) [31–33]. Early-onset CAD was defined as a manifestation of the disease symptoms at an age ≤55 years, according to the WHO MONICA (Multinational Monitoring of trends and determinants in Cardiovascular disease) [34]. Because less than 10% of the early-onset CAD cases who attended our Cardiology Department were women, only male patients were included in the study. The patients were recruited based on two inclusion criteria: they had suffered a first episode of CAD and they underwent a coronary angiography that confirmed the presence of at least one atherosclerotic coronary artery. A luminal narrowing > 70% was considered as indicative of atherosclerotic lesion. Controls were eligible residents and blood bank donors, all male and aged ≤65 years. The only inclusion criteria for these controls was the absence of a previous history of CAD-episodes, irrespective of the presence of hyperlipemia (including the treatment with lipid-lowering drugs), hypertension, or diabetes.

All the patients and controls were Caucasians from the region of Asturias and gave their informed consent to participate in the study, approved by the Ethical Committee of Hospital Central Asturias.

2.2. Definition of the analytical parameters

Blood was obtained from the patients during hospitalization and their analytical profile was determined. Type 2 diabetics were individuals with a clinical history of diabetes mellitus or who had a fasting blood glucose level > 125 mg/dl (7.0 mmol/L) in two different measures, according to the *American Diabetes Association criteria for classification and diagnosis of Diabetes* [35]. Patients and controls were defined as hypertensives if they had a documented history of hypertension, were receiving antihypertensive drugs, or had a systolic blood pressure > 140 mm Hg and/or a diastolic blood pressure > 90 mm Hg. Fasting blood values of total cholesterol, LDL-cholesterol, and triglycerides were also determined. Because some of the patients and controls were receiving lipid-lowering drugs these analytical levels would not reflect with reliability the basal lipid profile in the two groups. To determine the association between high cholesterol values and CAD we defined as hypercholesterolaemics individuals who received lipid lowering drugs or who had fasting total cholesterol > 220 mg/dL.

2.3. NF-KB variants genotyping

We genotyped three variants in the *NFKB1*, *NFKBIA*, and *NFKBIZ* genes. These variants were selected based on reported studies that described a positive association with disease-biological traits, such as cancer, psoriasis, arthritis, or coronary artery disease. The information related with these variants, including flanking sequence, reported population frequencies, and linkage disequilibrium (LD) values were obtained from the *Ensembl* web site (www.ensembl.org). All the variants were genotyped by polymerase chain reaction (PCR) amplification of genomic DNA with specific primer-pairs (Supplementary Table 1) followed by electrophoresis size fractioning of the corresponding fragment-alleles.

The *NFKB1* rs28362491 (-94 delATTG) was a common insertion/deletion (indel) polymorphism in the promoter region of *NFKB1*, and has been associated with differences in gene expression (the deletion would drive less promoter activity) (**13**). It has been widely studied in cancer and immune-mediated processes, including atherosclerosis and CAD. The DNAs were amplified with a 5´end fluorescence-labelled primer and the insertion/deletion alleles visualised by capillary electrophoresis (Life technologies) (Supplementary figure). The *NFKBIZ*

Table 1
Main values in the total patients and controls.

	Patients N = 609	Controls N = 423	P value	OR (95%CI)
Smoking	540 (89%)	141 (33%)	< 0.001	15.62 (11.40-21.73
Hypertension	241 (40%)	61 (15%)	< 0.001	3.81 (2.79-3.26)
Diabetes	97 (16%)	48 (11%)	0.04	1.48 (1.02-2.14)
Hypercholesterolemia	190 (31%)	93 (22%)	0.001	1.61 (1.21-2.14)
Cholesterol (mg/dl)	216 ± 35	203 ± 35	< 0.01	1.04 (1.01-1.06)
LDL (mg/dl)	136 ± 43	128 ± 36	< 0.001	1.03 (1.02-1.04)
Triglycerides (mg/dl)	165 ± 95	124 ± 69	< 0.001	1.05 (1.01-1.11)

indel genotyping rs3217713 (a 20 nt insertion/deletion polymorphism) was previously associated with the risk of Psoriasis [30]. PCRs were generated with a 5´end fluorescence-labelled primer visualised by capillary electrophoresis.

The *NFKBIA* rs8904 in complete LD with rs696, a variant in the 3´ untranslated region that would affect gene expression and activation of the NF-KB pathway [15,24]. We genotyped rs8904 instead of rs696 because the nucleotide changes in rs8904 affected a site for a restriction enzyme (*HhaI*), and was possible to genotype this SNP by a PCR-restriction fragment length polymorphism approach (PCR-RFLP) (Supplementary figure).

As a quality control of the genotyping method for the three variants we first confirmed the genotypes of several individuals by Sanger sequencing of PCR fragments. In addition, we included DNAs of known genotypes (and confirmed them) in all the PCR runs. In reference to the genotyping call-rate, the quality of all the DNAs was enough to render an amplification signal enough to define the genotypes of all the patients and controls (Supplementary figure). The absence of non-amplifiable samples was expected because most of the patients and controls had been successfully studied for other gene variants [31–33].

2.4. Statistical analysis

All the participant´s anthropometric, analytical and genetic data were collected in an Excel file. The values from the patient and control cohorts are available (anonymized for the identification of participants) upon request to the corresponding author.

Power calculation: because there was no data about the frequency of the three gene variants in our population, we first genotyped 200 controls (randomly chosen from the total 423 healthy controls) and determined the allele frequencies in our population (Supplementary Table 2). We confirmed that the genotype frequencies for each polymorphism did not deviate from the Hardy–Weinberg equilibrium (http://www.oege.org/software/hwe-mr-calc.shtml). The sample size required for a case-control study at a power of 80 and a confidence interval (95%CI) of 95% was calculated online with the *EpiTools* epidemiological calculator (http://epitools.ausvet.com.au). The predicted sample sizes for different odds ratios (ORs) are presented in the online supplementary material (Supplementary Table 3).

Genetic association study: The statistical analysis was performed with the free R-software (http://www.r-project.org). The Chi^2 test was used to compare allele and genotype frequencies between patients and controls, and the frequencies of smoking, diabetes, hypertension, and hypercholesterolemia between patients and controls. Odds ratios (OR´s) and the 95% confidence intervals (95%CI) were also calculated. Because we studied three gene variants, the standard $p < 0.05$ value was corrected by multiple testing and a $p < 0.01$ value was considered as statistically significant for the association between the gene variants and CAD. We also performed a multivariate logistic regression analysis (generalized linear model; LGM) including the genetic variants, smoking, diabetes, hypertension, and hypercholesterolemia to determine the association of these variables with the risk for CAD.

3. Results

In Table 1 we present the main anthropometric and analytic values in the early-onset CAD and healthy controls. All the individuals were male with a mean age of 49.28 (± 8.58; range 21–55 years) for patients and 60.81 (± 3.09; range 25–65 years) for controls. As previously reported, smoking was the strongest predictor for early-onset CAD in our population. The risk values for the other CAD-risk factors were significantly reduced compared to smoking.

Patients and controls were genotyped for the three common *NFKB1*, *NFKBIA* and *NFKBIZ* variants (Table 2). The preliminary analysis of 200 controls showed a MAF close to the reported for other Caucasian populations (Supplementary Table 2). We calculated the sample size required for a power of 80 assuming a 95% CI at OR values between 1.2 and 1.5. The total available allele number (n = 2,064, 609 patients + 423 controls) was enough to detect a difference in allele frequencies at ORs = 1.3 (*NFKB1* and *NFKBIA*) and 1.4 (*NFKBIZ*) (Supplementary Table 3).

Table 2
Genotype and minor allele frequencies (MAF) in the patients and controls. The genotype frequencies for the three variants did not deviate from the Hardy-Weinberg equilibrium.

		Patients N = 609	Controls N = 423
NFKB1 rs28362491 (-94 delATTG)	II	227 (37)	163 (39)
	ID	**291 (48)**	**201 (47)**
	DD	**91 (15)**	**59 (14)**
MAF: Del	**I**	**745 (0.61)**	**527 (0.62)**
Eur = 0.41	**D**	**473 (0.39)**	**319 (0.38)**
		Allele: p = 0.64, OR = 1.05 (0.88–1.26)	
*NFKBIZ** rs3217713	**II**	**319 (52)**	**262 (62)**
	ID	**248 (41)**	**140 (33)**
	DD	**42 (7)**	**21 (5)**
MAF: Del	**I**	**886 (0.73)**	**664 (0.78)**
Eur = 0.23	**D**	**332 (0.27)**	**182 (0.22)**
		Allele: p = 0.004, OR = 1.37 (1.11–1.68)	
NFKBIA rs8904	CC	**222 (35)**	**161 (38)**
	CT	**277 (46)**	**205 (48)**
	TT	**110 (19)**	**57 (14)**
MAF: T	**C**	**721 (0.59)**	**527 (0.62)**
Eur = 0.39	**T**	**497 (0.41)**	**319 (0.38)**
		Allele T, p = 0.17, OR = 1.14 (0.95–1.36)	

Eur = MAF in the Europeans, 1000 genomes.

* Deletion carriers (Ins/Ins vs. Ins/Del + Del/Del): p = 0.003, OR = 1.48 (95%CI = 1.15–1.90).

The genotyping of the 609 patients and 423 controls showed frequencies did not deviate from the expected under the Hardy-Weinberg equilibrium in the two groups. Moreover, allele frequencies in our controls were close to the reported in Europeans. We determined the association between the three NF-KB variants and early-onset CAD (Table 2). The only significant association was for the *NFKBIZ* rs3217713: the deletion allele was significantly more frequent in the patients than in controls (0.27 vs. 0.22; p = 0.004) with an OR = 1.37 (95%CI = 1.11–1.68). The two deletion genotypes were more frequent in the patients (ins/ins vs. ins/del + del/del, p = 0.003), with an OR = 1.48 (95%CI = 1.15–1.90).

We performed a multiple logistic regression (LGM) including hypercholesterolemia, type 2 diabetes, hypertension, smoking, and the *NFKBIZ* genotype as covariables (Table 3), and the rs3217713 deletion carriers remained significantly associated with early-onset CAD (p = 0.01). Thus, in our population the *NFKBIZ* variant was an independent risk factor for developing early-onset CAD. The distribution of diabetes, hyperlipemia and hypertension in patients and controls

Table 3
Summary of the multiple logistic regression (linear generalised model) including the *NFKBIZ* genotype (deletion-carriers), smoking, diabetes, hypertension, and hypercholesterolemia as covariables.

	Estimate	p-value	Exp. Estimate 2.5% / 97.5%
Intercept	0.16	$< 10^{-16}$	0.11 / 0.22
NFKBIZ ID + DD	1.43	0.01	1.14 / 1.96
Smoking	13.72	$< 10^{-16}$	9.91 / 10.24
Hypertension	3.01	$< 10^{-6}$	2.09 / 4.40
Diabetes*	1.18	0.48	0.75 / 1.87
Hypercholesterolemia*	1.13	0.49	0.79 / 1.61

* Diabetes and Hypercholesterolemia were correlated with Smoking in the bi-variable analysis, with smokers being significantly more hypercolesterolemics ($p < 0.001$) and diabetics (p = 0.02).

according to the smoking status showed a significant higher frequency of hypercholesterolemia among smokers in both, patients and controls (Supplementary Table 4). Also, smokers had higher mean cholesterol and LDL values in the two groups.

4. Discussion

The main finding of our study was the association between early-onset CAD and a common *NFKBIZ* variant. Recent studies identified *NFKBIZ* as an important player in the pathogenesis of cancer, psoriasis, and renal fibrosis [26–29,36]. *NFKBIZ* variants have been associated with the risk of developing psoriasis, a disease in which inflammation plays a fundamental role [30, 37].

The rs3217713 was an intronic indel without a recognised functional effect. However, is near to the intron-exon boundary and could thus be linked to differences in pre-mRNA splicing and the amount of the protein expressed [30]. *NFKBIZ* encodes a nuclear NF-KB inhibitor that regulates the LPS-induced IL-6 production [4]. Interestingly, IL-6 is a key mediator of the inflammatory process that has been associated with the progression of atherosclerotic plaque and plaque instability [38,39]. Moreover, the IL-6 mRNA levels are significantly increased in the atherosclerotic arteries compared to non-atherosclerotic vessels [40]. Based on these evidences, it could be speculated that IL-6 regulation is a potential link between *NFKBIZ* variation and CAD.

We did not confirm a significant association between CAD and two common *NFKB1* and *NFKBIA* variants. The *NFKB1* promoter indel has been linked to the risk for CAD by some authors [15–17,41]. *NFKBIA* variants were also associated with CAD in some studies [19,42]. However, at least one large-scale study found no association with *NFKBIA* rs696, a SNP in complete LD with rs8904 genotyped in our study (15). There are several differences between our cohorts and the studied by others that could explain the genetic association discrepancies. The most likely was the different ethnic background, but also the early-onset, the only-male composition, and the higher frequency of smokers among our patients. Also, our study was based on patients with angiographically confirmed atherosclerotic lesions in coronary arteries.

Finally, although the association between *NFKBIZ* variants was plausible in view of the biological role of the NF-KB components in atherosclerosis, we are well aware that our study was based on a limited number of individuals and would require confirmation in larger cohorts from different populations. In particular, for the *NFKBIA* T-allele we found a higher but non-significantly different frequency in the patients, and we could not rule-out an association with the risk of early-onset CAD in a larger population analysis. A limitation of our study was also the lack of a reported functional link between the studied variants and inflammation, and in particular for the *NFKBIZ* indel. This was an intronic variant that could affect RNA-splicing and the amount of the nuclear inhibitor, and this could in turn affect the expression of several inflammatory mediators, such as IL-6, and have an influence on the atherosclerotic plaque progression [4]. In addition to studies to determine differences between the two gene variants on *NFKBIZ* expression, other studies to determine whether the two variants were associated with differences in circulating levels of inflammatory molecules should be of interest.

5. Conclusions

In conclusion, we report the association between a common *NFKBIZ* indel polymorphism and early-onset CAD. Our study was based on only-male individuals, and patients with angiographically confirmed coronary lesions. The association between this gene and CAD was plausible considering the role of the NF-KB pathway on inflammation and atherosclerosis, but would require replication in other populations. In addition, studies focused to establish a functional effect of this *NFKBIZ* polymorphism should be of upmost interest.

Contributor ship

All the authors contributed to this work by recruiting the cohorts or performing the genetic and statistical analysis.

Competing interests

None of the authors have competing interests related to this work.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.imlet.2019.02.007.

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