ORIGINAL ARTICLES

■ ISCHEMIC HEART DISEASE

The involvement of the renin-angiotensin system gene polymorphisms in coronary heart disease

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Introduction and objectives. Previous studies angiotensin-converting enzyme gene insertion/deletion polymorphism ACE (I/D), angiotensinogen gene polymorphism, and angiotensin II AT1 receptor polymorphism in relation to coronary heart disease controversial results. This study was designed to analyze the association between these gene polymorphisms and the first coronary event in individuals residing on Grand Canary Island, Spain.

Patients and method. Case-control study. Case subjects (n=304) were recruited at the first coronary event; age-matched controls (n=315) were randomly selected from the Grand Canary population. Participants were examined for the usual risk factors. Blood samples were obtained for biochemical analyses and DNA extraction. Genotyping was performed by PCR and restriction analysis.

Results. Neither ACE (I/D) nor AT1 receptor polymorphism was associated with coronary heart disease, whereas the frequency distribution of AGT M235T genotypes among patients and control subjects (TT: 29% and 19%; MT: 48% and 50%; MM: 22% and 31%, respectively) was statistically different (P=.003). Multiple logistic regression analysis identified the TT genotype of the angiotensinogen gene (OR=1.9; 95% CI 1.1-3.4), diabetes (OR=4.4; 95% CI 2.0-9.4) and hypertension (OR=2.1; 95% CI, 1.3-3.3) as risk factors predicting the coronary event.

Conclusions. Our results provide no evidence of an association between ACE (I/D) or AT1 receptor polymorphism and coronary heart disease. However, homozygosity for the T allele of the angiotensinogen gene, diabetes and hypertension independently place individuals at higher risk of experiencing a coronary event on Grand Canary Island.

Key words: Angiotensinogen. Angiotensin-converting enzyme. Angiotensin II receptor. Coronary heart disease. Genes.

Relevancia de los polimorfismos génicos del sistema renina-angiotensina en la enfermedad coronaria

Introducción y objetivos. Estudios previos sobre la relación de la enfermedad coronaria y el polimorfismo de inserción/deleción de la enzima conversiva de la angiotensina (ECA [I/D]), el polimorfismo del gen del angiotensinógeno AGT M235T, o del receptor AT1 de la angiotensina II (A1166C) han demostrado resultados controvertidos. El objetivo de este estudio fue determinar la asociación entre estos polimorfismos génicos y el primer acontecimiento coronario en la población de Gran Canaria.

Pacientes y método. Estudio de casos y controles ajustados según edad. Los casos (n = 304) se seleccionaron al padecer un primer acontecimiento coronario; los controles constituyen una muestra aleatoria poblacional (n = 315). Todos los sujetos fueron evaluados para los factores de riesgo clásicos. Se tomaron muestras sanguíneas para determinaciones analíticas y extracción de ADN. Las genotipificaciones se realizaron por PCR y análisis de restricción.

Resultados. No se encontró asociación entre el polimorfismo ECA (I/D), AT1R (A1166C) y la enfermedad coronaria, mientras que la distribución de frecuencias de los genotipos del angiotensinógeno entre pacientes y controles (TT: 29 y 19%; MT: 48 y 50%; MM: 22 y 31%, respectivamente) resultaron estadísticamente diferentes (p = 0,003). El análisis multivariado identificó como factores predictores de acontecimiento coronario al genotipo TT del gen del angiotensinógeno (OR = 1,9; IC del 95%, 1,1-3,4), la diabetes (OR = 4,4; IC del 95%, 2,0-9,4) y la hipertensión (OR = 2,1; IC del 95%, 1,3-3,3).

Conclusiones. No se ha observado asociación entre el polimorfismo ECA (I/D), AT1R (A1166C) y la enfermedad coronaria. Sin embargo, la homocigosis TT del gen del angiotensinógeno, la diabetes y la hipertensión arterial predisponen de manera independiente a la aparición de un primer acontecimiento coronario en la población canaria.

Palabras clave: Angiotensinógeno. Enzima conversiva de la angiotensina. Receptor de la angiotensina II.

ABBREVIATIONS

AGT: angiotensinogen.
AT1R: AT1 receptor of angiotensin II
ACE: angiotensin-converting enzyme
BMI: body mass index
OR: odds ratio
PCR: polymerase chain reaction
RAAS: renin-angiotensin-aldosterone system

Enfermedad coronaria. Genes.

INTRODUCTION

Cardiovascular diseases–especially coronary artery disease–are the main cause of death in developed countries and their relation with certain risk factors like arterial hypertension, dyslipemia, smoking, or diabetes mellitus is well-known. It also is known that a family history of ischemic heart disease is a powerful independent coronary risk factor; this illustrates the multifactorial nature of the disease, in which environmental factors and genetic factors interact.[1]

New molecular biology techniques applied to genetic diagnosis make it possible to study the mechanisms underlying individual and familial predisposition to suffering certain diseases. Specifically, in relation to coronary artery disease, the genetic markers linked to the renin-angiotensin-aldosterone system (RAAS) have received special attention, not only because of their well-known effects on vascular homeostasis,[2,3] but also the promise of the use of angiotensin-converting enzyme inhibitor (ACEI) to reduce morbidity and mortality in ischemic heart disease.[4,5]

The findings that relate RAAS genes to ischemic heart disease are recent and several polymorphisms in different genes of this system have been described.[6-8] I/D polymorphism of the gene that codes for ACE consists of the presence (I) or absence (D) of 287 base pairs in intron 16 of the gene.[6] The D allele is accompanied by higher plasma and tissue concentrations of ACE activity than the I allele,[6,9] which could provide a pathophysiological explanation for the higher incidence of myocardial infarction and coronary artery disease in individuals with the DD genotype.[10] Various studies have tried to confirm these observations, with debatable results.[11-14]

The angiotensinogen gene, a second component of the RAAS, has been related with the progression of heart disease. A polymorphism in exon 2 of the gene, consisting of the substitution of methionine by treoni-ne in position 235 of the coded protein (M235T),[7,15] has been associated with higher angiotensinogen concentrations,[7,16] higher blood pressure,[17] and even the development of pre-eclampsia.[18] A study made in Spain by our group has reported an association between the TT genotype and increased risk of coronary artery disease, independently of the presence of arterial hypertension.[19]

A third component of the RAAS is the gene of the AT1 receptor (*AT1R*) of angiotensin II. Most of the effects of angiotensin II are mediated through *AT1R*.[8] A polymorphism of receptor AT1 (A1166C) has been identified in region 3´ of the gene that corresponds with an A#-?C substitution in messenger RNA. The association between this polymorphism and coronary artery disease has been analyzed by different groups.[20,21]

In this study we examined the classic risk factors and various gene polymorphisms of the RAAS that may explain the high incidence of coronary artery disease in the Canary Islands.[22]

PATIENTS AND METHOD

Subjects

A study was made of cases and controls adjusted for age who met the following requirements: age over 18 years, Canary Island origin (born in the Canary Islands into a family with at least three previous generations of Canary Islanders) and absence of cardiovascular antecedents, contagious infectious diseases, and parenteral drug use.

The cases (n=304; mean age 56±10 years; 237 men) were selected consecutively in 1996-1998 after suffering a first coronary event (angina or acute myocardial infarction), which was diagnosed by clinical and electrocardiographic criteria and confirmed by coronary angiography.

The controls (n=315; mean age 54±10 years; 223 men) were selected from the census lists of different municipalities of Gran Canaria Island and the sample was stratified by a multi-stage conglomerate. In this group, the presence of previous cardiovascular events was excluded by a clinical examination.

All subjects completed a questionnaire with a trained nurse (AAP) that included demographic data: age, place of birth, habitat, hypertension, diabetes mellitus, smoking, alcohol use, physical activity, and use of oral contraceptives or hormonal replacement therapy by women. Blood pressure measurements were made in triplicate using a semiautomatic monitor (Omron Hem 705 CP), which has been homologated and validated by the British Society of Arterial Hypertension.[23] Arterial hypertension was defined as 140/90 mm Hg or more. The body mass index (BMI) was calculated as weight/height[2] and obesity was defined as a BMI over 26. Smokers were defined as anyone who had smoked
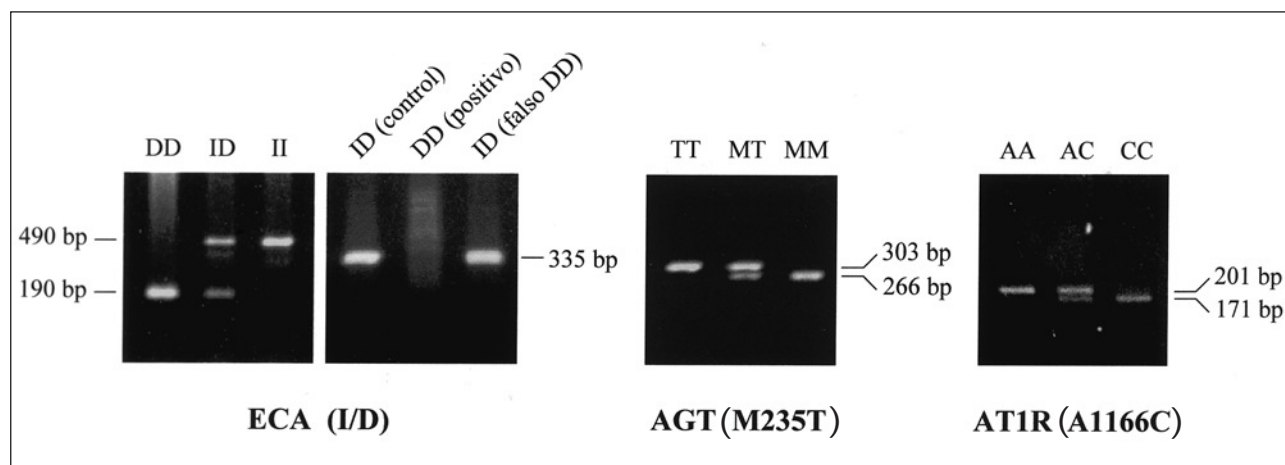
Fig. 1. Visualization of PCR products by ethidium bromide staining according to the genotypes of ACE (I/D), AGT M235T, and AT1R (A1166C) polymorphisms. False DD homozygotes were identified using specific primers for insertion (second panel): an ID control sample (lane 1) and two DD samples, as determined in the first round of PCR (lanes 2 and 3), were submitted to a second amplification cycle. The 335-bp band in lane 3 after reamplification indicates the existence of the I allele (false DD).

in the last year. Several blood samples were obtained, after a 12 h fast, for biochemical determinations and DNA extraction and at least 2 urine samples were obtained for albuminuria determination.

Laboratory methods

Plasma concentrations of glucose, creatinine, total cholesterol, HDL-C, and triglycerides were determined by enzymatic-colorimetric methods. The LDL-C was calculated using the Friedewald formula. Lp(a) was analyzed by immunoturbidimetry (Boehringer, Mannheim). Homocysteine was determined by polarized fluorescence immunoanalysis, which had a variation in the intra-assay coefficient of 1.9% (Abbott, Diagnostic Division). Microalbuminuria was measured by immunoturbidimetric analysis (Boehringer, Mannheim).

The genomic DNA used for the genetic determinations was extracted from leukocytes using a standardized procedure. The I/D genotypes of ACE were determined following the procedure described by Rigat[24] in 1992. Briefly, a reaction was made with 10 pmol of each primer in a final volume of 25 μl. DNA was amplified 30 cycles with denaturation at 94°C for a minute, ringed at 58°C for a minute and extension at 72°C for 2 min. PCR generated a fragment with 190 base pairs (bp) in the case of deletion, and a 490-bp fragment in the case of the insertion.

Since the D allele is amplified in mainly heterozygotic samples,[25] each sample of the DD genotype of ACE was reamplified in a second round of PCR with specific primers for insertion.[13] An amplification protocol described previously[26] was used and ID and II samples were used as amplification controls. Our rate of DD false positives was only 1.2% compared with 4-5% described in other published studies.

The M235T polymorphism of AGT was analyzed by the *mismatch* method described previously,[15] with some modification. Genomic DNA was amplified by initial denaturation at 94°C followed by 30 cycles at 94°C for one minute, at 66°C for one minute, and at 72°C for one minute and a final extension at 72°C for 2 min. The 10 μl of amplified product was digested overnight at 37°C with 0.3 units of *Sfa*NI. A total of 579 subjects for which a serology library and gene library were available were gene typed for the A1166C polymorphism of the *AT1R* gene. Genotypes A1166C in region 3′UTR of gene *AT1R* were determined following the protocol described by Nakauchi, et al.[27] Amplification yielded a 201-bp product. The mutated product (A1166C) generated 171-bp and 30-bp fragments when cut with *HaeIII*. The products were developed with an ethidium bromide stain after electrophoretic fractionation through agarose gels (Figure 1).

Statistical analysis

The SPSS statistical program version 8.0 for Windows was used for data analysis. Differences were considered statistically significant if $P<.05$. All continuous variables were expressed as mean ± standard deviation. The Student t test was applied to independent samples to compare means between two groups and χ^2 analysis was used to compare differences in the distribution of genotypes and other coronary risk factors, including sex, arterial hypertension, diabetes mellitus, smoking and alcohol use, and habitat and sedentary life style between patients and the control group.

The Kolmogorov-Smirnov test was used to estimate the normal distribution of variables. The odds ratio (OR) and 95% confidence intervals (CI) were calculated to estimate the risk of coronary artery disease associated with categorized continuous variables (age <50

TABLE 1. **Population characteristics**

	Controls (n = 315)	Cases (n = 304)	P
Age (years)	54 ± 10	56 ± 10	.06
Urban habitat	182 (81%)	209 (88%)	.005
Smoking habit	86 (27%)	152 (51%)	< .001
Alcohol (g/day)	11 ± 19	16 ± 27	.007
Arterial hypertension	103 (33%)	147 (49%)	.001
Systolic blood pressure (mm Hg)	135 ± 26	135 ± 24	.95
Diastolic blood pressure (mm Hg)	84 ± 12	76 ± 13	< .001
Diabetes mellitus	36 (11.5%)	103 (34%)	< .001
Glycemia (mg/dl)	105 ± 27	112 ± 50	.03
BMI (kg/m^2)	27.3 ± 3.8	27.2 ± 3.7	.8
Total cholesterol (mg/dl)	232 ± 40	198 ± 43	< .001
LDL (mg/dl)	151 ± 36	138 ± 37	< .001
HDL-C (mg/dl)	50 ± 13	35 ± 9	< .001
Total cholesterol/HDL-C	4.9 ± 1.4	5.9 ± 1.7	< .001
Triglycerides (mg/dl)	151 ± 77	124 ± 67	< .001
Lp(a) (mg/dl)	36 ± 43	54 ± 68	< .001
Homocysteine (mol/l)	15.7 ± 7.3	14.6 ± 5.2	.03
Microalbuminuria (mg/g creatinine)	27 ± 135	43 ± 150	.17
Creatinine (mg/dl)	0.87 ± 0.31	0.81 ± 0.22	.01
BMI: body mass index			

χ^2 = V; squared

or =50 years; BMI <26 or = 6; alcohol use <30 or = 30 g/day; systolic blood pressure <40 or = 140 mm Hg; diastolic blood pressure <90 or = 90 mm Hg; total cholesterol = 200 or >200 mg/dl; HDL-C = 65 or >65 mg/dl; LDL-C = 160 or >160 mg/dl; triglycerides = 150 or >150 mg/dl; total cholesterol/HDL-C ratio = 5 or >; Lp(a) = 30 or >30 mg/dl; glycemia = 126 or >126 mg/dl; creatinine<1 or = 1 mg/dl; homocysteine = 15 or >15 μmol/l, and microalbuminuria <30 or = 30 mg/g creatinine) and for discrete variables: diabetes yes/no, rural/urban habitat, hypertension yes/no, sex, smoking yes/no and sedentary life style yes/no. The Hardy-Weinberg equilibrium for the frequencies of ACE, AGT and AT1R genotypes was tested by χ^2 analysis. The OR and 95% CI were calculated to estimate the relative risk of coronary artery disease associated with the ACE (I/D), AGT M235T, and AT1R (A1166C) polymorphisms. Finally, the independent variables with predictive capacity for coronary artery disease were determined by logistic regression analysis.

RESULTS

Univariate analysis

Study of patients, life style, and cardiovascular risk factors

This study included 619 patients selected at random (460 men and 159 women), ranging in age from 25 to 80 years. All the patients completed the study protocol. In Table 1 the main characteristics of the population, separated by cases and controls, are described. Male sex, urban habitat, smoking, arterial hypertension, and diabetes were significantly more frequent factors in the group with coronary artery disease. The control group had significantly higher HDL-C values. The groups did not differ significantly in age, BMI, systolic blood pressure, or albuminuria. Nevertheless, the diastolic blood pressure, total cholesterol, and LDL-C were lower in patients than in the control group, which probably reflects the use of antihypertensive and lipid-lowering medication as routine treatment. Coronary patients had a significant increase in the total choleste-rol/HDL-C ratio and plasma Lp(a). Paradoxically, plasma homocysteine concentrations were higher in the control group than in patients, although both values were within the range of normality.

Distribution of the ACE, AGT, and AT1R genotypes between cases and controls

For the ACE (I/D), AGT M235T, and AT1R (A1166C) polymorphisms, it was confirmed that the proportion of genotypes fit the Hardy-Weinberg equilibrium.

The distribution of the frequencies of the ACE, AGT, and AT1R genotypes for the polymorphisms mentioned is described in Table 2. In our population, the frequency of the ACE DD genotype was higher (Table 3) than the frequency reported for populations of diverse ethnic origin, including other Caucasian populations.[13,28-33] Nevertheless, the frequency of the DD genotype was not significantly greater among coronary

TABLE 2. **Distribution of the ACE, AGT, and AT1R genotypes**

	Controls	Cases	χ^2	P
ACE genotype				
DD	137 (43.5)	128 (42.7)		
ID	132 (41.9)	140 (46.7)		
II	46 (14.6)	32 (10.6)		
Total	315	300	2.69	.26
AGT genotype				
TT	59 (18.7)	87 (29.1)		
MT	157 (49.9)	145 (48.5)		
MM	99 (31.4)	67 (22.4)		
Total	315	299	11.61	.003
AT1R genotype				
AA	150 (49.7)	133 (48.0)		
AC	126 (41.7)	122 (44.0)		
CC	26 (8.6)	22 (7.9)		
Total	302	277	.34	.844

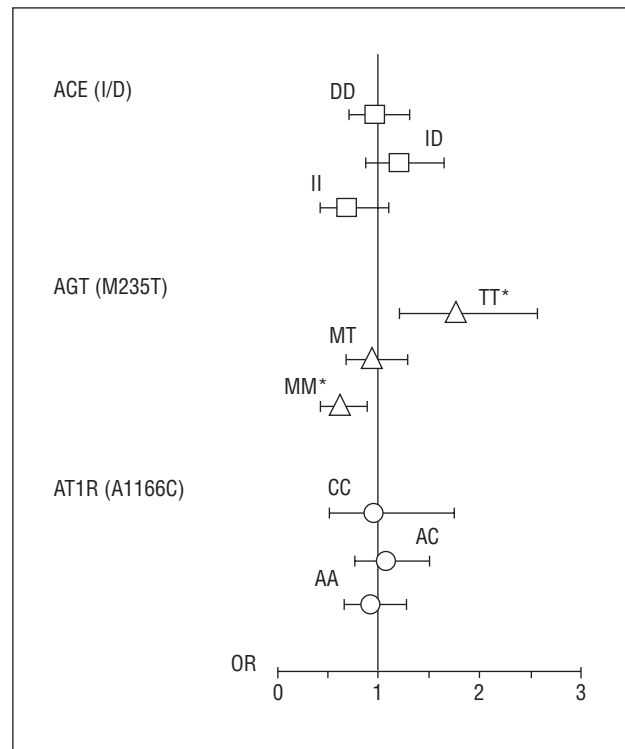The figures in parenthesis indicate the percentage of the total.

Fig. 2. Estimation of the risk of coronary artery disease related with genetic polymorphisms of the angiotensin-converting enzyme (ACE I/D), angiotensinogen (AGT M235T), and AT1R (A1166C) genes. OR indicates odds ratio. *P<.05.

patients.

Examination of the distribution of frequencies in the AGT M235T polymorphism revealed a significant difference (X^2=11.60; *P*=.003) between coronary patients and controls. The frequency of the TT homozygote was significantly higher (X^2=9.08; *P*=.002) in cases than in controls. There were no differences in the distribution of the frequencies of AT1R polymorphisms between patients and the control group. The risk (OR) of coronary artery disease among individuals with the genotypes analyzed are shown in Figure 2. The analyses adjusted for age revealed no significant changes for the ACE (I/D) and AT1R (A1166C) polymorphism. Nevertheless, the risk associated with TT homozygosis was 1.78 (95% CI, 1.22-2.59; *P*<.05), whereas participants with the MM genotype had an OR of 0.63 (95% CI, 0.43-0.90; *P*<.05). No significant differences between heterozygotic subjects were observed.

Multivariate analysis

Step by step analysis of multiple logistic regression identified individuals at risk of presenting a coronary event. Two vectors of variables were established, one using the genotypes and the other with traditional coronary risk factors. Using 50% as the cut-off point, the model correctly classified 78% of the participants and 76% of coronary patients. The sensitivity of the model was 74% and the specificity was 80%.

In multiple logistic regression analysis adjusted for different risk factors, the TT genotype presented an OR of 1.9 (95% CI, 1.06-3.40; *P*=.03). The highest estima-

TABLE 3. **Distribution of ACE genotypes in different countries**

Region	Control group (n)	Genotype			Author and bibliographic reference
		DD (%)	ID (%)	II (%)	
Japan	76	18,3	48,9	32,8	Mizuiri et al[32]
South Asia*	442	18,3	41,8	39,8	Sagnella et al[31]
Chile	117	18,5	49,0	32,5	Jalil et al[28]
Australia	51	22,0	53,0	25,0	Smith et al[33]
France	157	30,6	44,0	25,4	Marre et al[29]
U.S.A.	2,340	30,9	49,2	19,9	Lindpaintner et al[13]
Germany	234	33,0	50,0	17,0	Schmidt et al[30]
Gran Canaria	315	43,5	41,9	14,6	Este estudio

*The study of Sagnella, et al. Includes different ethnic groups.

ted risks corresponded to diabetes (OR=4.4; 95% CI, 2.0-9.4; *P*<.001), total cholesterol/HDL-C ratio (OR=4.0; 95% CI, 2.4-6.8; *P*<.001), smoking (OR=2.7; 95% CI, 1.7-4.3; *P*<.001), alcohol use (OR=2.2; 95% CI, 1.2-4.2; *P*=.02), and hypertension (OR=2.1; 95% CI, 1.3-3.3; *P*=.001). There was no statistical evidence indicating that any genotype of ACE (I/D) or AT1R (A1166C) polymorphism could improve the capacity to predict a coronary event with respect to the traditional predictive factors cited above.

DISCUSSION

The basis of genetic approaches to complex diseases has been reviewed by Risch[34] in 1996, and other authors. Together with the problems derived from sample size and ethnic diversity, there is a certain tendency to publish studies in which a positive association has been demonstrated, a bias that makes it difficult to analyze association studies.[14,35] An additional characteristic of our study was that it minimized the effect of the selection bias and loss of patients –due to early mortality–since it was a consecutive population studied during the acute phase of the coronary event.

Cambien, et al.[10] were the first to describe the association between the DD genotype of the ACE gene and myocardial infarction, finding a more marked association between subjects with a low coronary risk profile according to classic risk factors. In other studies, the DD genotype has been related with coronary artery disease,[27] coronary restenosis,[36,37] and a family history of myocardial infarction.[11] In contrast, no evidence was obtained that the genetic variations in the I/D polymorphism of the ACE gene influence the incidence of coronary artery disease in our population in a relevant way. Similarly, in the prospective study of Lindpaintner[13] in 1995, no significant association was found between this polymorphism and ischemic heart disease, although a lower frequency of coronary events in II homozygotes was appreciated. We are found a higher than expected frequency of the DD genotype in both the patients who had suffered a coronary episode and the control population, compared with previous studies,[28,29,31] including the study made in Spain by Espinosa, et al.,[38] who found the DD genotype in 33.9% of the control population.

The TT genotype of the angiotensinogen gene was present in 19% of our control population, compared with only 15% in most Western populations.[7] Our results demonstrated a strong association between this polymorphism and the risk of coronary artery disease. Thus, TT homozygotes had a risk that was approximately two-fold,[19] whereas the M235 homozygotes had a lower risk of coronary artery disease. Although studies of association suggest a role of the AGT gene in hypertensive disease, the link between the AGT gene and essential hypertension initially described by Jeunemai-

tre et al.[7] has not been reproduced in other studies.[39,40] In our analysis, no relation was found between the M235T polymorphism of the angiotensinogen gene and hypertension, which indicates that this polymorphism constitutes a coronary risk factor independent of the degree of arterial hypertension or antihypertensive treatment.

Multivariate analysis of logistic regression confirmed the TT genotype of angiotensinogen as a marker that identifies individuals with a two-fold greater probability of developing coronary artery disease, independently of the presence of other environmental risk factors.

Some studies of association have analyzed the risk profile of suffering a coronary event for combinations of polymorphic variants of certain RAAS genes.

Tiret, et al.[20] described a synergic association of DD (I/D) homozygotes of the ACE and CC gene of the A1166C polymorphism of the *AT1R* gene. For these authors, this gene interaction of risk for myocardial infarction might suggest a possible epistatic effect of the two genes, assuming that the C allele of *AT1R* is associated with a modified response of the angiotensin II receptor, thus modulating the possible risk conferred by the D allele of the ACE gene. Álvarez, et al[21], in a young Spanish population, developed a case-control study to determine the I/D genotypes of the ACE gene and A1166C of the gene of receptor 1 of angiotensin II. Separately, no association was observed; but the ACE-DD and AT1R-CC genotypes interacting synergically were associated with coronary artery disease (OR=5.32; 95% CI, 1.45-19.51). In contrast, Gardemann, et al.[41] found no synergic interaction between the polymorphic variants of the ACE and AT1R genes and coronary artery disease in 2244 Caucasian men. Similarly, Rice, et al.[42] demonstrated the absence of a synergic association with myocardial infarction, although they found a weak association between some genotypes (AC/II and CC/DD) and coronary stenosis. Coinciding with these studies, we found an association between AT1R (A1166C) polymorphism and coronary ischemic disease. Similarly, we found no epistatic effects between the polymorphic variants of the ACE and AT1R genes (data not shown).

Our work confirmed that diabetes mellitus–which has a prevalence of 12% in the control group–was an important risk factor for coronary artery disease, and was related to genetic and environmental factors in the Canary Island population. In addition, our population had some of the highest total cholesterol and LDL-C levels, as well as the peculiarity of being a region with a high rate of smoking.[43] Nevertheless, the prevalence of arterial hypertension did not differ from that of other Spanish or European regions.[44]

The relation between the TT genotype of angiotensinogen and ischemic heart disease found in our study, if confirmed in more extensive studies and the rest of the

Spanish population, could have special interest with respect to the application of more aggressive preventive measures in this subpopulation.

CONCLUSIONS

The CC genotype of the AT1 receptor of angiotensin II and the DD genotype of angiotensin-converting enzyme did not behave like predictors of coronary artery disease. Nevertheless, TT homozygosis of the angiotensinogen gene predisposed independently to the appearance of a first coronary episode in the Canary Island population.

ACKNOWLEDEMENTS

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