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Variants translating reduced expression of the beta estrogen receptor gene were associated with increased carotid intima media thickness

A cross-sectional study in late postmenopausal women

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Abstract

There is debate on the role of estrogens in modulating the risk for atherosclerosis in women. Our purpose was to investigate whether the size of the estrogenic impact was independently associated with variation of carotid intima-media thickness (IMT) in healthy late postmenopausal women. The levels of circulating estrogens have been used in previous studies but the influence of SNPs of the estrogen receptors (ER) α and β have not been investigated.

We performed a crossed-sectional study of 91 women in a university hospital. We used a double approach in which, in addition to the measurement of estradiol levels by ultrasensitive methods, genetic variants (SNPs) associated with differing expression of the ER α and β genes were assessed. Multivariable analysis was used to examine the association of candidate factors with the value of IMT and plaque detection at both the carotid wall and the sinus.

A genotype combination translating reduced gene expression of the *ER*β was directly associated with IMT at both the carotid wall ($P = .001$) and the sinus ($P = .002$). Other predictors of IMT were the levels of glucose, positively associated with IMT at both the carotid wall ($P < .001$) and the sinus ($P = .001$), age positively associated with IMT at the sinus ($P = .003$), and levels of vitamin D, positively associated with IMT at the carotid wall ($P = .04$).

Poorer estrogenic impact, as concordant with a SNP variant imposing reduced expression of the *ER*β, was directly associated with IMT at both the carotid wall and the sinus. Glucose level, vitamin D only for the carotid wall, and age only for the sinus, also emerged as independent factors in the IMT variance.

Abbreviations: AIC = Akaike's Information Criterion, BMI = body mass index, CHAFEA = Consumers, Health, Agriculture and Food Executive Agency., CVD = cardiovascular disease, DNA = deoxyribonucleic acid, E2 = estradiol, EDTA = ethylenediaminetetraacetic acid, ELISA = enzyme-linked immunosorbent assay, ER = estrogen receptor, FSH = follicle-stimulating hormone, HDL-C = high-density lipoprotein cholesterol, HOMA = homeostasis model assessment, HWE = Hardy-Weinberg Equilibrium, IMT = intima-media thickness, LDL-C = low-density lipoprotein cholesterol, MAF = minor allele frequency, NTC = Non template control, PCR = polymerase chain reaction, SD = standard deviation, SNP = single nucleotide polymorphism, SWAN = Study of Women Across the Nation, TC = total cholesterol, UTR = untranslated region, VIF = variance-inflation factor.

Keywords: atherosclerosis, carotid IMT, estrogens, postmenopausal women, risk factors

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1. Introduction

Cardiovascular disease (CVD) is the principal cause of mortality and morbidity in women.[1,2] The disease exhibits specific features in the female, and interest has arisen about whether the influence of risk factors is modified, or even whether the disease may have some distinct risk factors in women.[3,4]

There is a debate on whether the extent of the estrogenic impact may be a key factor modulating cardiovascular risk. For example, premature natural or surgical menopause is associated with increased cardiovascular risk.[5,6] The responsibility of hormonal changes associated with menopause has been investigated by measuring the association between endogenous estrogens, usually estradiol (E2), and cardiovascular events in observational studies. But results have been conflicting, with both protective[7,8] and neutral roles.[9,10] The weakness of studies assessing estrogenicity on the basis of only the circulating E2 levels resides in the physiological oscillations of the hormone. Moreover, accurately measuring very low levels of E2, as in post-menopause, represents a technical challenge. Genetic variants (single nucleotide polymorphisms, SNPs) of the estrogen receptors (*ER*), α and β, which may condition changes in the hormonal message at the tissue level, define an innovative and improved approach. Genetic changes reflect variants that are operative along the whole life, and therefore may translate into considerable difference in the accumulated estrogenic impact. Taking advantage of this approach, we have focused our study in postmenopause, which represents the wide life period in which atherosclerotic lesions develop and in which risk for cardiovascular events starts being significant.

The objective of the present study was to disclose whether estrogenicity, which in our hands translated an in-depth analysis including the circulating levels of E2 and the SNP variants of both the *ER* α and β genes, was associated with variation in the degree of subclinical atherosclerosis in a group of late postmenopausal women. Subclinical atherosclerosis was assessed at the carotid artery by measuring the intima media thickness (IMT) of the far wall in the carotid artery and in the carotid sinus.

2. Materials and methods

2.1. Study design and patients

We designed a crossed study in which one hundred postmenopausal women were invited to participate when coming for their regular health control at our center. The postmenopausal status was confirmed by at least one-year amenorrhea or a surgically induced menopause, together with follicle-stimulating hormone (FSH) levels $\geq$ 30 mIU/mL and E2 levels within the postmenopausal range. Women were considered eligible if of Caucasian ethnicity, if they were free of any previous or current clinical chronic disease, including CVD, osteoporotic fracture, cancer, or cognitive disease, and had never used menopausal hormonal therapy. Each woman was assessed only once for each of the planned explorations, which were scheduled within the interval of one month.

The institutional review board at our centre (Hospital Clínico Universitario) approved the study and written informed consent was obtained from each woman.

2.2. Study measures

Women were explored according to a protocol designed to analyze a group of variables related with atherosclerotic risk.

2.3. Anthropometric, clinical and biochemical assessments

Women were measured their height, mass and waist circumference, and the body mass index (BMI) was calculated as the ratio between mass (kg) and square height (m^2). Blood pressure was measured in the left arm using an automatic blood pressure monitor (Omron M6, HEM-7001-E, Omron Healthcare Co., Ltd. Kyoto, Japan) and expressed in mmHg. Mean arterial pressure was calculated as diastolic pressure plus 1/3 pulse pressure, where pulse pressure was systolic pressure minus diastolic pressure.[11] Blood was drawn between 08.00–10.00 a.m. after an overnight fast, and the serum separated. A routine analysis of basic biochemical parameters and a complete lipid profile were performed using enzymatic methods with an autoanalyzer (Olympus AV 5200; Tokyo, Japan).

The levels of the circulating hormones were measured by immunoassay. FSH (mIU/mL) was quantified by chemiluminescence (BioMérieux Inc., Hazelwood, MO, USA, intra- and inter-assay coefficients of variation $\leq$ 10.0%); E2 (pg/mL) was measured using an ultra-sensitive (<1.4 pg/mL) commercial solid phase enzyme-linked immunosorbent assay (ELISA) based on competitive binding (DRG International, Springfield, NJ, USA, intra- and inter-assay variation coefficients $\leq$ 10.0%). Insulin (μIU/mL) was measured by the C-peptide ELISA kit (IBL International GMBH, Hamburg, Germany, intra- and inter-assay coefficients of variation $\leq$ 6.7% and $\leq$ 10.0%, respectively). Vitamin D was quantified with the Elecsys vitamin D total assay (Roche Diagnostics International, Totkreuz, CH), which measures 25-hydroxyvitamin D by an electro-chemiluminescence binding procedure. The Elecsys coefficients of variation were $\leq$ 6,5% (intra) and $\leq$ 11.5% (inter-assay). The insulin resistance index (homeostasis model assessment, HOMA) was calculated as fasting serum insulin in μIU/mL x (fasting serum glucose in mg/dL x 0.05551)/22.5).[12]

2.4. SNPs analyses

Blood samples were collected in tubes with anticoagulant (disodium-ethylenediaminetetraacetic acid, EDTA) and kept refrigerated at 4° C. Nucleated cells were used for deoxyribonucleic acid (DNA) isolation with a genomic DNA extraction kit (REAL; Durviz, Valencia, Spain) after lysis of red blood cells with ammonium chloride (10 mM $KHCO_3$, 150 mM NH_4Cl, 0.1 mM EDTA-Na_2, pH 7.4). The 260/280 absorbance ratio of the product ranged from 1.6–2.0, indicating high-quality DNA.[13]

The TaqMan SNP Genotyping Assay (Applied Biosystems, Foster City, CA) on a 7900 HT Fast Real-Time polymerase chain reaction (PCR) System (Applied Biosystems) was used to get the allelic discrimination for genotyping of SNPs: *rs2234693*, *rs9340799*, *rs3798577* and *rs61112218* in the *ESR1* (*ERα*) gene; and *rs1256030* and *rs4986938* in the *ESR2* (*ERβ*) gene. We followed the protocol provided by the manufacturer in which, briefly, 20 ng of genomic DNA was amplified in the presence of 1 x TaqMan probe assay and 1 x TaqMan Universal PCR Master Mix (Applied Biosystems). The 7900 HT thermocycler software was employed for allelic discrimination. Reproducibility was estimated by re-genotyping 5–7% of samples in each plate and was > 99%. About 0.5% of the genotypes were ambiguous and samples had to be re-genotyped.

2.5. Imaging assessments

IMT was understood as the area of tissue starting at the luminal edge of the artery and ending at the boundary between the media

and the adventitia. Both the right and the left carotid artery were explored by B-mode ultrasound with the help of the QLAB-IMT program integrated into a Philips HD-11 XE Scanner. This machine was fitted with a lineal probe with an emission frequency capable of being modulated within a range of 3 to 12 MHz. QLAB-IMT allows an automated measurement of the IMT, with the help of a program that has been specifically designed for escaping from the potential errors entailed in the manual position of cursors. An experimented ultrasonographer, who followed the standards for image acquisition established at the Manheim consensus,[14] performed the examination procedure. The IMT values were obtained in segments free of plaque from 2 locations, the far wall of the common carotid artery at approximately 1 cm proximal to the carotid sinus, and the far wall of the carotid sinus. The mean of the values at both the right and the left carotid arteries was used for analyses.

Carotid plaques were assessed in consistence with the criteria of the Manheim consensus, i.e., a focal structure that encroaches into the arterial lumen of at least 0.5 mm or 50% of the surrounding IMT value or demonstrates a thickness 11.5 mm as measured from the media-adventitia interface to the intima-lumen interface.[14]

2.6. Statistical analysis

Three multivariable models have been carried out using stepwise forward and backward direction based on Akaike's Information Criterion (AIC).[15] Multiple linear regression was applied to detect linear relationships between dependent variables carotid or sinus IMT and multiple logistic regression for the presence and absence of plaque. Age, BMI, waist perimeter, mean arterial pressure, E2, FSH, triglycerides, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), total cholesterol (TC), vitamin D, glucose, insulin, and HOMA index were included as quantitative independent variables and SNPs, treated as dummy variables, which were codified as 0 or 1. The SNPs were grouped into two categories according to the inheritance model using the SNPStats software.[16] This software was also used to estimate allelic and genotypic frequencies and to determine if they met the Hardy-Weinberg Equilibrium (HWE).

Collinearity was assessed according to variance-inflation factor (VIF)[17] using Car package.[18] Normality in residuals for both multiple linear regressions was explored using Shapiro–Wilk's test.

R software (3.6.2)[19] was used for all analysis and *P* value cutoff for significance was set at.05.

3. Results

3.1. Baseline data

The group was composed of 91 out of the 100-screened women because 8 participants refused ultrasonographic examination and technical difficulties impaired a satisfactory assessment in one participant (Fig. 1). Table 1 shows the anthropometric, clinical and biochemical parameters of the participants who completed the protocol.

The mean age of the participants was 61.8 years and the mean menopausal age was 10.8 years; 21 women (23%) had suffered surgical menopause. There were 10 women who smoked, 24 women who had a BMI > 30 kg/m^2, and 42 women with some hypertensive feature (systolic blood pressure ≥ 140 mm Hg, diastolic blood pressure ≥ 90 mm Hg, or use of anti-hypertensive medication). Also in Table 1, women in the cohort had slight overweight and hypercholesterolemia, although the HDL-C levels were high enough to yield a normal mean TC/HDL-C ratio. Additional features of interest were that women had low mean levels of E2, as corresponding to an advanced postmenopausal period, a normal mean HOMA index, and reasonable mean vitamin D levels. Allelic frequencies were similar to those described in the 1000 Genomes database for Europeans (Table 2),

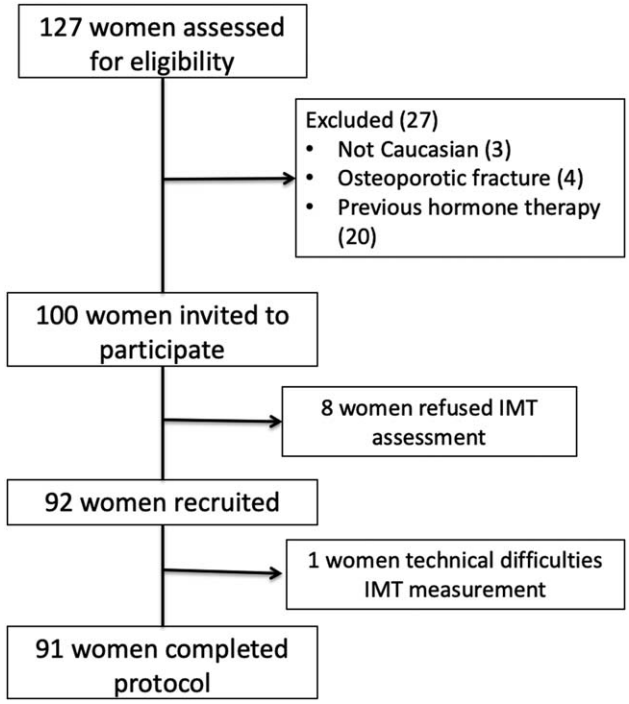

Figure 1. Flowchart of the study showing details of participants' numbers at each stage of the study.

Table 1

Anthropometric, clinical and biochemical parameters of patients.

Parameter	Mean	SD
Age (yr)	61.8	7.2
Years since menopause	10.8	7.8
BMI (Kg/m^2)	27.9	4.3
Waist perimeter (cm)	89.0	11.5
Mean arterial pressure (mm Hg)	103.5	13.9
TC (mg/dL)	208.5	24.6
HDL-C (mg/dL)	64.9	15.2
LDL-C (mg/dL)	122.8	24.0
TC/HDL-C ratio	3.4	0.9
Triglycerides (mg/dL)	103.7	51.0
FSH (IU/L)	74.6	34.1
Estradiol (pg/mL)	5.0	4.9
Vitamin D (ng/mL)	25.0	9.5
HOMA index	2.7	2.1

BMI = body mass index, FSH = follicle stimulating hormone, HDL-C = high-density lipoprotein cholesterol, HOMA = homeostasis model assessment, LDL = low-density lipoprotein cholesterol, TC = total cholesterol.

Table 2

Single nucleotide polymorphisms (SNPs) that were investigated.

SNP	Chromosome	Gene	Position *	Location	Major allele	Minor allele	MAF	*P*-HWE
rs2234693	6	*ESR1*	151842200	Intron variant	T	C	0.49	.51
rs9340799	6	*ESR1*	151842246	Intron variant	A	G	0.40	.82
rs61112218	6	*ESR1*	151941318	Intron variant	A	T	0.09	.54
rs3798577	6	*ESR1*	152099995	3'-UTR variant	T	C	0.41	.37
rs4986938	14	*ESR2*	64233098	3'-UTR variant	C	T	0.36	.64
rs1256030	14	*ESR2*	64280452	Intron variant	G	A	0.47	.52

* Genomic coordinates according to Genome Reference Consortium Human Build 38 patch release 12 (GRCh38.p12).
HWE = Hardy–Weinberg equilibrium, MAF = Minor allele frequency, UTR = Untranslated region.

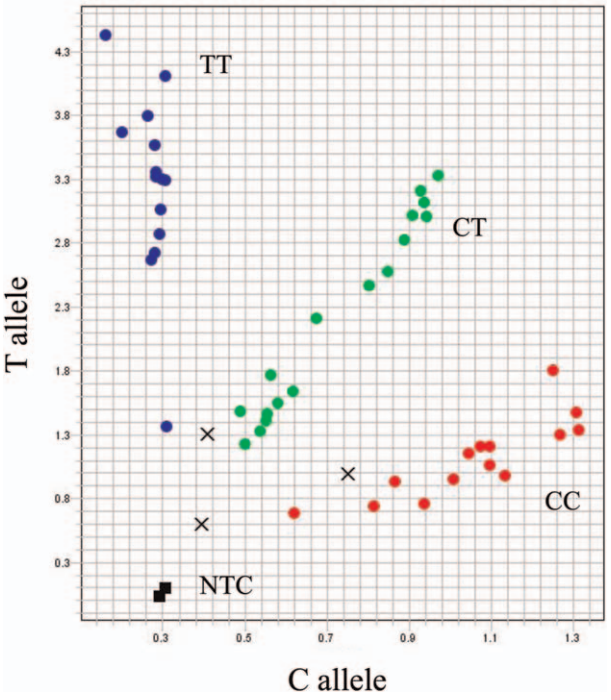

Figure 2. A representative allelic discrimination plot for the *rs4986938* SNP in the *ESR2* gene is shown. NTC = Non template control. CC, CT, and TT are the different genotypes for *rs4986938* SNP. Crosses represent samples of undetermined genotype.

and genotypic frequencies met the HWE indicating a correct genotyping. A representative plot showing the allelic discrimination for the rs4986938 SNP in the *ESR2* gene is presented in Figure 2.

3.2. Carotid IMT

The mean (standard deviation, SD) IMT values at the carotid wall and in the carotid sinus were 0.672 (0.096) mm and 0.716 (0.122) mm, respectively. Atheromatous plaques were found in 10 women.

Simple linear regression showed no significant correlation between the circulating level of E2 and IMT at either the carotid wall or the sinus (Supplemental Digital Figure S1, http://links.lww.com/MD/G176). Multiple linear regression analysis was used with carotid IMT and sinus IMT as dependent variables (Table 3). Both the levels of glucose ($P < .001$) and of vitamin D ($P = .04$) were positively associated with IMT in the carotid wall. The SNP analysis showed that the genotype TT of the SNP *rs4986938* of the *ERβ* gene, which is associated with lower expression of the gene and therefore interpreted as a reduction of function,[20] was directly associated with carotid IMT ($P = .001$).

The IMT at the sinus also exhibited a positive association with the level of glucose ($P = .001$). Age also emerged as a positively associated factor ($P = .003$) at this territory. As for the carotid wall, the SNP genotype TT of the SNP *rs4986938* of the *ERβ* gene was directly associated with sinus IMT ($P = .002$).

Model for presence/absence of plaque did not converge due to low number of presence.

Table 3

Best model multiple linear regression analysis for carotid artery and sinus according to AIC.

Dependent variable	Independent variables	Estimate	Standard error	*t*-value	*P*-value	Adjusted R^2	AIC
Carotid wall	(Intercept)	0.074	0.133	0.557	.582	0.599	−186.53
	rs3798577 (ESR1)	−0,045	0,027	−1.683	.103		
	rs61112218 (ESR1)	−0.049	0,029	−1.700	.100		
	rs4986938 (ESR2)	0.138	0.037	3.771	.001		
	Age	0.003	0.002	1.715	.097		
	Glucose	0.003	0.001	4.690	<.001		
	Triglycerides	<0.001	<0.001	1.431	.164		
	Vitamin D	0.002	0.001	2.211	.035		
Carotid bulb	(Intercept)	−0.493	0.233	−2.120	.043	0.539	−157.99
	rs3798577 (ESR1)	−0.052	0.038	−1.376	.179		
	rs4986938 (ESR2)	0.185	0.054	3.413	.002		
	Age	0.009	0.003	3.306	.003		
	BMI	0.008	0.005	1.605	.119		
	FSH	0.001	0.001	1.461	.155		
	Glucose	0.004	0.001	3.662	.001		

AIC = Akaike's Information Criterion, BMI = body mass index, *ESR1* = estrogen receptor 1, *ESR2* = estrogen receptor 2, FSH = follicle stimulating hormone.

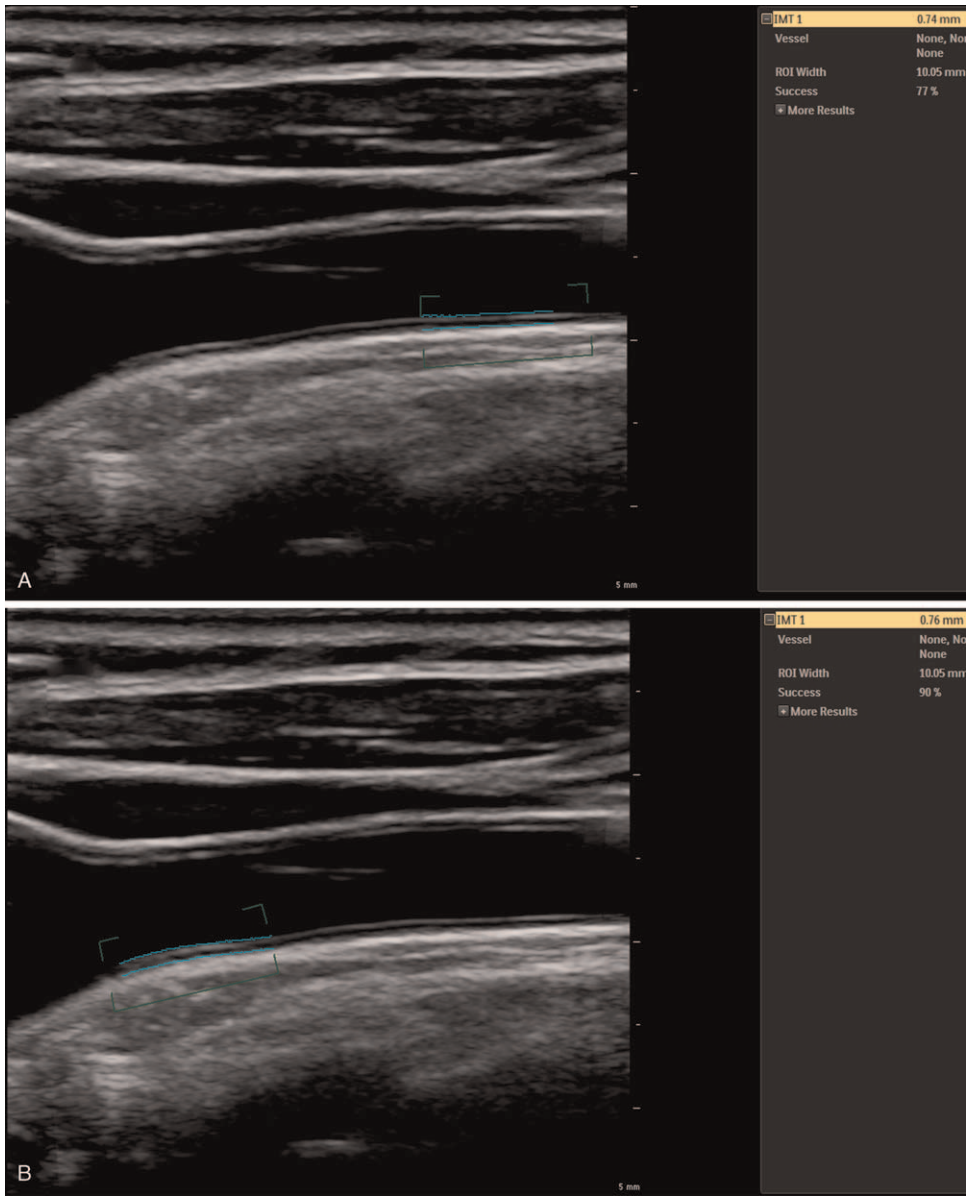

Figure 3. Representative B-mode ultrasound images showing the IMT values corresponding to the carotid wall (0.74 mm, panel A) and the sinus (0.76 mm, panel B) of one woman with the TT genotype at the *rs4986938* SNP in the *ESR2* gene. Both values were in the higher range of the IMT results obtained in the cohort.

Representative carotid wall and sinus B-mode ultrasound images corresponding to one woman with the TT genotype at the *rs4986938* SNP in the *ESR2* gene are shown in Figure 3.

4. Discussion and conclusion

Our study focused on a group of women with a mean age of 61.8 years, a period poorly investigated in studies trying to enlighten the role of estrogens in the development of atherosclerosis. That stage, however, is crucial to elucidate the impact of estrogens on atherogenesis, because the lesions of the disease start being detectable during that life period.

The findings of our study are particularly engaging in that regard. We addressed the issue following a double strategy. Firstly, we measured the circulating level of E2 assuming that differences at the precise time-point of our study were representative of a distinct status of estrogenicity in the long haul. Indeed, and although this may be arguable, this has been until now the only approach in studies investigating the impact of endogenous estrogens on CVD in postmenopausal women[7–10] or on other outcomes, like for example breast cancer.[21] We found that E2 did not emerge as an independent variable with an impact on IMT at either the carotid wall or the sinus in our analysis. In contrast, we found that the genotype TT of the SNP *rs4986938* of the *ERβ* gene, which is associated with lower expression of the gene,[20] was directly linked with carotid IMT at both the carotid wall ($P = .001$) and the sinus ($P = .002$). This finding is important because it is compatible with a persistently reduced estrogenic function at the target level along the whole life. So, it may be taken as an indication in favor of a protective effect of estrogens in the long-term.

Similar to our study, Finnish investigators could not find an association of IMT with circulating E2 in a cohort of similar age to our group.[22] Other studies have detected some associations between endogenous estrogens and the atherosclerosis burden, but when focusing on the menopausal transition. In a subset of perimenopausal women participating in the Study of Women Across the Nation (SWAN) study, Wildman et al[23] found an inverse association between declining levels of E2 and the adventitia diameter of the common carotid artery. Similar findings were reproduced when a cohort of pre- and perimenopausal women was followed longitudinally for a median of 3.7 years[24] or in women suffering a more rapid menopausal transition.[25] Our study now adds another piece of evidence in that the small, but persistent, changes represented by differences in the functional effect of estrogens at the tissue level may translate into IMT variation.

We also explored the impact of a list of other candidate cardiovascular variables on IMT. Glucose emerged as a predictor at both the carotid wall and the carotid sinus. The impact of glucose on cardiovascular risk has been previously reported in several studies.[26,27] Also, age appeared as an independent predictor of IMT at the sinus. In contrast, other recognized risk factors, such as the lipid profile, BMI, mean arterial pressure, waist perimeter or insulin resistance did not come out as independent predictors in our analysis. While the case of insulin resistance may be interpreted as partly embedded within the impact of glucose, the limited size of our cohort may be argued to explain the reduced sensitivity for identifying other independent candidates. Another limitation of this study is the cross-sectional design, which prevents the acquisition of evidence on a temporal relationship between estrogen exposure and IMT variation.

This reinforces the value of our findings concerning the estrogenic action, which showed a significant association with IMT even in conditions in which the independent effect of other recognized predictors of atherosclerosis was undetectable. The case of vitamin D merits a specific comment because the direct association with carotid IMT is against findings in observational studies.[28] However, randomized controlled trials have been unable to demonstrate a beneficial action of vitamin D supplementation on cardiovascular outcomes.[29]

To conclude, poorer estrogenicity, as measured by the SNP variant imposing reduced expression of the *ERβ*, was directly associated with IMT at the two explored territories, carotid wall and sinus.

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Funding acquisition: Antonio Cano.
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Validation: Antonio-Jorge Cano-Marquina, Miguel-Ángel García-Pérez, Alicia M Maceira, Juan Cosín-Sales.
Writing – original draft: Antonio Cano.
Writing – review & editing: Miguel-Ángel García-Pérez, Juan J Tarín, Antonio Cano.

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