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Apolipoprotein E-ε2 and Resistance to Atherosclerosis in Midlife: The PESA Observational Study

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BACKGROUND: APOE is a known genetic contributor to cardiovascular disease, but the differential role *APOE* alleles play in subclinical atherosclerosis remains unclear.

METHODS: The PESA (Progression of Early Subclinical Atherosclerosis) is an observational cohort study that recruited 4184 middle-aged asymptomatic individuals to be screened for cardiovascular risk and multiterritorial subclinical atherosclerosis. Participants were *APOE*-genotyped, and omics data were additionally evaluated.

RESULTS: In the PESA study, the frequencies for *APOE* -ε2, -ε3, and -ε4 alleles were 0.060, 0.844, and 0.096, respectively. This study included a subcohort of 3887 participants (45.8±4.3 years of age; 62% males). As expected, *APOE*-ε4 carriers were at the highest risk for cardiovascular disease and had significantly greater odds of having subclinical atherosclerosis compared with ε3/ε3 carriers, which was mainly explained by their higher levels of low-density lipoprotein (LDL)-cholesterol. In turn, *APOE*-ε2 carriers were at the lowest risk for cardiovascular disease and had significantly lower odds of having subclinical atherosclerosis in several vascular territories (carotids: 0.62 [95% CI, 0.47–0.81]; P=0.00043; femorals: 0.60 [0.47–0.78]; P=9.96×10^{-5}; coronaries: 0.53 [0.39–0.74]; P=0.00013; and increased PESA score: 0.58 [0.48–0.71]; P=3.16×10^{-8}). This *APOE*-ε2 atheroprotective effect was mostly independent of the associated lower LDL-cholesterol levels and other cardiovascular risk factors. The protection conferred by the ε2 allele was greater with age (50–54 years: 0.49 [95% CI, 0.32–0.73]; P=0.00045), and normal (<150 mg/dL) levels of triglycerides (0.54 [0.44–0.66]; P=4.70×10^{-9} versus 0.90 [0.57–1.43]; P=0.67 if ≥150 mg/dL). Omics analysis revealed an enrichment of several canonical pathways associated with anti-inflammatory mechanisms together with the modulation of erythrocyte homeostasis, coagulation, and complement activation in ε2 carriers that might play a relevant role in the ε2's atheroprotective effect.

CONCLUSIONS: This work sheds light on the role of APOE in cardiovascular disease development with important therapeutic and prevention implications on cardiovascular health, especially in early midlife.

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GRAPHIC ABSTRACT: A graphic abstract is available for this article.

Key Words: atherosclerosis ■ cardiovascular risk ■ cholesterol ■ midlife ■ omics

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Novelty and Significance

What Is Known?

- APOE plays a major role in lipid transport and metabolism, contributing to the risk of developing cardiovascular disease (CVD).
- The *APOE*-ε4 allele is a risk factor for atherosclerosis, the main precursor of CVD, which may be mediated by lipid levels.
- The effect conferred by the *APOE*-ε2 allele on cardiovascular health remains unclear, depending on age, sex and other cardiovascular risk factors.

What New Information Does This Article Provide?

- Middle-aged *APOE*-ε2 carriers present lower odds of having subclinical atherosclerosis in multiple vascular sites (carotid, femoral, aorta and coronary arteries), compared with ε3/ε3 carriers.
- The atheroprotection conferred by ε2 alleles is mostly independent of LDL (low-density lipoprotein)-cholesterol levels, except in females and younger individuals, opening a window for prevention strategies in early midlife.
- *APOE*-ε2 carriers present an enrichment of several canonical pathways associated with anti-inflammatory mechanisms together with the modulation of erythrocyte homeostasis, coagulation and complement activation that might play a relevant role in the ε2's atheroprotective effect.

APOE contributes to the development of atherosclerosis in part due to the differential interaction of APOE isoforms with lipids. However, the mechanisms underlying this differential effect are intricate and warrant further investigation in midlife. To that end, we investigated the odds of having multiterritorial subclinical atherosclerosis across *APOE* genotypes and across age, sex, and triglycerides groups. We found that ε4 carriers have higher odds of generalized subclinical atherosclerosis, which was mainly mediated by higher levels of LDL-cholesterol. We also observed consistently lower odds of having subclinical atherosclerosis across multiple vascular territories (carotid, femoral, aorta, and coronary arteries) in ε2 carriers. This ε2's atheroprotection revealed to be mainly independent from low levels of LDL-cholesterol, except in females and younger individuals (age, 40–44 years). Moreover, ε2 carriers with high triglyceride levels (≥150 mg/dL) did not present such protection, suggesting the role of APOE on atherogenesis might be triglycerides-dependent and could also be modified early in midlife. Our omics data further indicated that ε2 carriers present an enrichment of several canonical pathways associated with anti-inflammatory mechanisms together with the modulation of erythrocyte homeostasis, coagulation, and complement activation that might play a relevant role in the ε2's atheroprotective effect. Our results open a venue for therapeutic strategies in cardiovascular health management.

Nonstandard Abbreviations and Acronyms

CACS	coronary artery calcium score
CVD	cardiovascular disease
CVRF	cardiovascular risk factor
FHS	Framingham Heart Study
GSEA	gene set enrichment analysis
HDL	high-density lipoprotein
LDL	low-density lipoprotein
PESA	Progression of Early Subclinical Atherosclerosis
SBP	systolic blood pressure
SCORE2	systematic coronary risk evaluation
VLDL	very low-density lipoprotein
VUS	vascular ultrasound

Apolipoprotein E plays a major role in lipid transport and metabolism, contributing to the risk of developing cardiovascular disease (CVD).[1] The *APOE* gene has 3 common alleles–ε2, ε3, and ε4–each differing in frequency within the White population (8%, 79%, and 13%, respectively).[1] The combination of these alleles gives rise to 3 homozygous (ε2/ε2, ε3/ε3, and ε4/ε4) and 3 heterozygous genotypes (ε2/ε3, ε2/ε4, and ε3/ε4). Despite differing in only 2 amino acid positions, these isoforms interact variably with specific lipoprotein receptors, impacting the clearance of chylomicron remnants and VLDL (very-low-density lipoprotein) from the bloodstream.[2] While the *APOE*-ε4 allele is generally associated with higher LDL (low-density lipoprotein)-cholesterol levels, the *APOE*-ε2 allele is often linked to reduced LDL-cholesterol, although it may result in elevated triglyceride levels due to impaired lipoprotein clearance.[1,3,4]

The ε4 allele has been associated with an increased risk of coronary artery disease and greater carotid atherosclerosis compared with the ε3/ε3 genotype.[4–7] In contrast, ε2 carriers generally exhibit lower carotid atheroma thickness, fewer atheroma plaques, and reduced coronary artery calcium score (CACS), all indicators of atherosclerosis.[4,8–10] The mechanisms behind these associations are complex and not entirely understood. For instance,

the increased cardiovascular risk associated with the ε4 allele may be mediated by lipid levels[11] or by the coexistence of other cardiovascular risk factors (CVRFs).[12] On the other hand, the atheroprotective effect of the ε2 allele seems to be multifactorial, with some studies attributing it to lower LDL-cholesterol levels[13] and others suggesting it may be independent of traditional CVRFs.[8,9,14] To add to this complexity, some ε2 homozygotes present high circulating triglyceride levels, which, when combined with other CVRFs, can increase the risk of developing type III hyperlipoproteinemia and CVD.[15,16]

Given the unclear influence of age, sex, comorbidities, and other CVRFs across studies, the role of APOE in CVD development is intricate and warrants further investigation, especially during early asymptomatic stages. In this context, the PESA (Progression of Early Subclinical Atherosclerosis) study aims to explore the earliest stages of atherosclerosis and its associated risk factors in a large cohort of middle-aged (40–54 years) asymptomatic individuals.[17] Our study intends to compare CVD risk and the presence of multiterritorial subclinical atherosclerosis across *APOE* genotypes in this cohort. Primarily, we hypothesize that the subclinical atherosclerosis burden will be lower in *APOE*-ε2 carriers and greater in ε4 carriers in comparison to the ε3/ε3 genotype. We also examine how age, sex, and triglyceride levels modify the impact of APOE on subclinical atherosclerosis. Through advanced imaging and omics data, we aim to uncover the molecular and cellular pathways that may contribute to the differential genetic predisposition conferred by APOE to early stage atherosclerosis, with special emphasis on identifying the atheroprotective mechanisms linked to the ε2 allele.

METHODS

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request and with a research proposal that will require further review and approval by the PESA Scientific Committee.

Study Design

This study was conducted within the baseline visit of the PESA study, which enrolled 4184 asymptomatic White individuals aged 40 to 54 years between 2010 and 2014. All volunteers were employees of the Santander Bank in Madrid, Spain. PESA participants were screened for atherosclerosis and CVRFs, with the aim of describing the prevalence and causes of subclinical atherosclerosis and its associated risk factors. The exclusion criteria included having CVD, cancer, or any disease expected to shorten the lifespan or influence protocol adherence. The detailed design has been reported previously.[17,18]

The PESA study (NCT01410318) was approved by The Ethics Committee of the Instituto de Salud Carlos III in Madrid (Spain), and all participants provided written informed consent.

APOE Genotyping

Blood samples were drawn during the baseline visit and stored at −80 °C until processed. DNA extraction from peripheral blood was performed as previously described.[19] *APOE* genotyping was performed by real-time PCR.[20] Real-time PCR reactions were loaded into 384 well plates using a Fluent liquid handling platform (Tecan Trading AG, Männedorf, Switzerland). Three independent reactions were performed to genotype each sample, 1 for each *APOE* allele (ε2, ε3, ε4) and each reaction well included: 40 ng of genomic DNA, 1X TaqMan Universal Master-Mix (Thermo Fisher Scientific), *APOE* primers, and *APOE*-FAM probe at a final concentration of 0.5 µM each, and ACTB (β-actin) primers and ACTB-VIC probe at a final concentration of 0.1 µM each. The final reaction volume was 10 µL. Negative controls were run in parallel for each primer and probe combination, and all reactions were performed in duplicate. Real-time PCRs were performed in a Biorad CFX-384 (BioRad, Hercules, CA) with the following amplification protocol: 95 °C for 10 minutes followed by 55 cycles with denaturation at 95 °C for 15 seconds, and annealing/extension at 64 °C for 1 minute. ΔCt values for each well were calculated by subtracting the Ct value for the control (ACTB) from the Ct value for the *APOE*. ΔCt values separated reactions in 2 clearly differentiated groups (positives and negatives). The phenotype for each sample was calculated by combining the results of the 6 ΔCt values generated (3 alleles X 2 replicates).

To confirm the *APOE* genotype obtained by real-time PCR, a subset of 156 PESA participants also underwent PCR-restriction fragment length polymorphic analysis, following published protocols.[21] Briefly, 40 ng of genomic DNA was amplified using the Platinum II Hot Start Green PCR Master-Mix (Invitrogen) kit with the primers ε2mut and ε3, as previously described.[21] The ε2mut primer differs from the genomic sequence in 1 base, allowing the introduction of a polymorphism, which is translated into a new recognition site for the AflII enzyme. In addition, the *APOE* gene sequence originally contains several recognition sites for the HaeIII enzyme. Thus, the double digestion of the amplified DNA with these restriction enzymes gives rise to a unique pattern for each allele according to the different combinations of the 3 isoforms of the *APOE* gene (ε2, ε3, ε4), allowing the identification of the 6 genotypes (ε2/ε2, ε2/ε3, ε2/ε4, ε3/ε3, ε3/ε4, ε4/ε4). Double digestion was performed in a final volume of 20 µL, using a concentration of 5 U of the AflIII and 1.5 U of the HaeIII enzymes (New England Biolabs) at 37 °C overnight. The analysis of the DNA fragments was done in low-temperature polymerization agarose 4% gels (LM Sieve, Conda, Madrid, Spain).

Assessment of CVRFs and CVD risk

The cardiovascular profile in the PESA study was determined from medical interviews, body measurements, and blood samples, as previously described.[17] Definition of traditional CVRFs follows established cutoffs: dyslipidemia as total cholesterol ≥240 mg/dL, LDL-cholesterol ≥160 mg/dL, HDL (high-density lipoprotein) cholesterol <40 mg/dL, or use of lipid-lowering drugs; diabetes as fasting plasma glucose ≥126 mg/dL or treatment with insulin or oral hypoglycemic medication; hypertension as systolic blood pressure (SBP) ≥140 mm Hg, diastolic blood pressure ≥90 mm Hg or use of antihypertensive medication; and smoking as being currently a smoker or having a lifetime consumption of >100 cigarettes.[22] High triglyceride levels were defined as triglycerides ≥150 mg/dL.[23]

Cardiovascular risk was assessed with 2 standard risk scales commonly used in clinical practice to identify individuals who should undergo prevention. First, the 30-year-FHS (Framingham Heart Study) scale was calculated to predict the long-term risk of developing CVD in 30 years, using the following variables: age, sex, total cholesterol, HDL-cholesterol, body mass index, SBP, hypertension, smoking, and diabetes.[24] Participants were classified as low risk if 30-year-FHS≤10%, moderate risk if 10%<30-year-FHS<20%, and high risk if 30-year-FHS≥20%. While this is one of the most used risk scales in research studies, the recently published systematic coronary risk evaluation (SCORE2) prediction algorithm has been calibrated to the 40- to 69-year-old European population, likely providing more accurate CVD risk estimates in the middle-aged Spanish PESA cohort.[25] SCORE2 measures the short-term risk of developing CVD in 10 years and uses the following variables: age, sex, total cholesterol, HDL-cholesterol, SBP, and smoking. Low/moderate risk was defined as SCORE2<2.5% if participants were younger than 50 years or SCORE2<5% if they were 50 years or older; and high/very high risk as SCORE2≥2.5% if participants were younger than 50 years or SCORE2≥5% if they were 50 years or older.[25]

Assessment of Subclinical Atherosclerosis

Subclinical atherosclerosis was determined by assessing the presence of atherosclerotic plaque and CACS. The imaging protocol has been described previously.[17] High-resolution 3-dimensional-vascular ultrasound (3D-VUS) was performed in the carotid and femoral arteries with a standardized 6-cm transducer for improved plaque detection.[26] Ultrasound studies were analyzed with QLab10.2 (Philips Healthcare, Bothell, WA). Plaque in any territory was defined as a focal protrusion into the arterial lumen of thickness >0.5 mm; >50% of the surrounding intima-media thickness; or a diffuse thickness >1.5 mm measured between the media-adventitia and intima-lumen interfaces.[22] Second, coronary artery calcium was detected with a 16-slice computed tomography scanner (Philips Brilliance; Philips Healthcare, Andover, MA) using noncontrast prospective electrocardiography-gated acquisition. CACS was calculated by the Agatston method, according to the protocol.[22] Imaging quality was visually inspected by experienced physicians.

Subclinical atherosclerosis measures considered in this study included 3D-VUS carotid, femoral and total plaque presence, CACS, and the previously reported PESA score,[22] which accounts for the multiterritorial extent of subclinical atherosclerosis based on the number of vascular sites affected identified by 2D-VUS (right and left carotid, right and left iliofemoral, abdominal aorta) and CACS≥1 (coronary arteries). The PESA score classified participants as disease-free (0 vascular sites affected) or as having focal (1 site), intermediate (2–3 sites), or generalized (4–6 sites) atherosclerosis.[22]

Omics Substudy Design

The PESA omics subcohort, for which proteomics, metabolomics, and methylomics analyses were performed, included 480 PESA participants divided into 2 groups: 240 individuals with higher burden of subclinical atherosclerosis (as measured by 2D-VUS) and 240 with lower burden, matched by age (±3 years), sex and traditional CVRFs (diabetes, smoking, dyslipidemia, and hypertension, prioritizing the matching by this order).[19,27] In addition, transcriptomics data from 36 PESA participants were retrieved from a pilot omics study. Furthermore, new transcriptomics data were generated to enrich the omics subcohort with 42 additional individuals with ε2/ε2 (n=9) and ε4/ε4 (n=33) genotypes, and 20 samples were resequenced from previous RNA-seq experiments to account for batch effects. From these 558 PESA individuals with transcriptomics data, *APOE* genotype information was available for 543, forming the final transcriptomics subcohort used in this study. Of those, methylomics data could be analyzed for 382, as previously described.[19]

Omics Data Generation

For methylomics, blood samples were collected, and DNA was extracted and processed as previously reported.[19] Briefly, 1 µg of high-quality DNA was used for methylome analysis at the CEGEN (Madrid, Spain). First, genomic DNA was subjected to bisulfite conversion with the EZ DNA Methylation Kit (Zymo Research). Subsequently, converted DNAs were processed to be hybridized in the Infinium MethylationEPIC BeadChip following the manufacturer's protocols (Illumina), and arrays were scanned using iScan System (Illumina). *Grim* epigenetic age acceleration was calculated from methylomics data as the difference between chronological age and epigenetic age estimated based on the *Grim* epigenetic clock.[19]

For transcriptomics, peripheral blood samples were collected in PAXgene Blood RNA tubes (Preanalytix), stored at −80 °C after collection, and processed as previously described.[19] Briefly, total RNA was automatically extracted using a QIAsymphony SP liquid handling robot and the QIAsymphony PAXgene blood RNA kit (Qiagen), following the manufacturer´s instructions. RNA quantity and integrity were measured using a NanoQuant Plate (Tecan) and a 2100 Bioanalyzer (RNA6000 Nano LabChip, Agilent), respectively. Two hundred nanograms of total RNA from samples with an RNA integrity number >6 were used to generate barcoded RNA-seq libraries using the NEBNext Ultra RNA Library preparation kit (New England Biolabs). The size and concentration of the libraries were checked with the Agilent 2100 Bioanalyzer DNA 1000 chip. On average, every 11 libraries with different barcodes were equimolar pooled, and concentration was determined using the Qubit fluorometer (Life Technologies). Library pools were sequenced on a HiSeq2500 (Illumina) to generate 60 bases of single reads. FastQ files for each sample were obtained using CASAVA v1.8 software (Illumina). For the new transcriptomics data (42 new samples and 20 resequenced samples), total RNA was used to generate barcoded RNA-seq libraries using the NEBNext Ultra II Directional RNA Library preparation kit (New England Biolabs) according to manufacturer's instructions. Libraries were then pooled and sequenced on a NextSeq 2000 (Illumina) to generate 60 bases single reads, which were then processed, including gene expression quantification from identified transcripts by RSEM v1.2.20, with genome reference GRCh38.82 using bowtie2 v2.2.5. Expected counts for each run were joined into a table with 43186 genes. Genes with expression value in CPM>1 for 80% of participants were selected and 12062 genes remained for normalization with the TMM method from the EdgeR package.

Statistical Analysis

Obtained frequencies of the *APOE* genotype were tested for Hardy-Weinberg equilibrium. *APOE* genotypes were grouped into ε2 carriers (including ε2/ε2 and ε2/ε3), ε3/ε3 carriers,

and ε4 carriers (including ε3/ε4 and ε4/ε4). ε2/ε4 carriers were excluded from the main analyses for clarity purposes.[28]

The distribution of continuous variables was assessed with graphical methods. Normally distributed continuous variables are expressed as mean±SD, whereas non-normally distributed variables are expressed as median (Q1–Q3). Categorical variables are expressed as N (%). Linear trends across *APOE* genotypes were evaluated with an extension of the nonparametric Wilcoxon rank-sum test. To analyze the interplay between *APOE* genotype, CVD risk, and subclinical atherosclerosis, a logistic ordinal model was used to evaluate the PESA score (independent variable) across *APOE* groups as a continuous variable (ε2/ε2+ε2/ε3>ε3/ε3>ε3/ε4+ε4/ε4), including an interaction term between *APOE* and CVD risk groups (30-year-FHS or SCORE2). For multivariate analysis, logistic regression models were used to assess the association between *APOE* genotypes and the main atherosclerosis measures (presence of any plaque, presence of carotid plaque, presence of femoral plaque, CACS>0 and PESA score) adjusting by relevant covariables following 3 different multivariate models: model 1: adjusted for age and sex; model 2: adjusted for age, sex, LDL- and HDL-cholesterol, SBP, diastolic blood pressure, smoking, diabetes, and use of lipid-lowering drugs; and model 3: adjusted for the same covariables as model 2 except for LDL-cholesterol, to assess its possible mediating effect. Logistic binary models were used for plaque and CACS (presence or absence) as dependent variables, whereas a logistic ordinal model was used for the PESA score (disease-free, focal, intermediate, or generalized subclinical atherosclerosis). For the multivariate analyses, we considered the *APOE* gene has an additive effect, and the number of alleles of each participant (ε2=0, 1, 2 and ε4=0, 1, 2) was included in the models. ε3/ε3 was used as reference. In addition, prespecified stratified analyses by sex, age (40–44, 45–49, and 50–54 years old), and triglycerides (<150 or ≥150 mg/dL) based on previous literature were developed. Results were expressed as the odds ratio and its 95% CI.

As for omics data, the *Grim* epigenetic age acceleration was compared between ε2 carriers versus the other *APOE* genotypes using a nonparametric 2-sample Mann-Whitney *U* test. Additionally, estimates for the intrablock correlation for duplicated transcriptomics samples with participant ID as block were calculated and then differential gene expression by empirical Bayes moderation of linear modeling of *APOE* genotypes with age, sex, and batch as covariates was computed using Limma-Voom R package.[29] The logFC obtained from the differential gene expression analysis comparing ε2 carriers versus the other *APOE* genotypes were used as rank criteria for a preranked gene set enrichment analysis (GSEA) with default scoring schema using c5.go.bp.v7.5.1 MSigDB gene set collection by GSEA-cli.sh from Broad Institute GSEA version 4.2.3. The false discovery rate *q*-value was calculated as previously described,[30] and functions with a false discovery rate <10% were selected as significant, following GSEA guidelines. Enriched pathways and genes of interest were represented using the GOPlot R package.[31]

All these statistical analyses were performed using Stata 18 (StataCorp, College Station, Texas) and R 4.2.0 (specific R packages are indicated throughout the Methods section). Differences were considered statistically significant at $P<0.05$.

RESULTS

Demographics and *APOE* Genotyping of the PESA Cohort

The original PESA cohort enrolled a total of 4184 asymptomatic middle-aged individuals. Of those, 94 participants were excluded due to problems in the DNA extraction protocol and 67 participants did not give consent to use blood samples (Figure 1). Thus, *APOE* genotyping was performed in 4023 PESA participants, of which 543 underwent RNA-Seq and 382 of those had additional methylomics data.[19] *APOE* genotyping results are shown in Figure S1A through S1C. As expected, the ε3 was the most common allele, with a frequency of 0.844, followed by the ε4 and ε2 alleles with frequencies of 0.096 and 0.060, respectively. Hence, the ε3/ε3 genotype was the most abundant (71.4%), followed by ε3/ε4 (15.9%) and ε2/ε3 (10.1%), and being ε2/ε4 (1.3%), ε4/ε4 (1.0%), and ε2/ε2 (0.3%) the less frequent *APOE* genotypes. Our results followed the Hardy-Weinberg equilibrium rule with no significant differences detected between the observed and the expected values for each *APOE* genotype (Figure S1D). These *APOE* genotyping results are in agreement with other Spanish cohorts.[32,33]

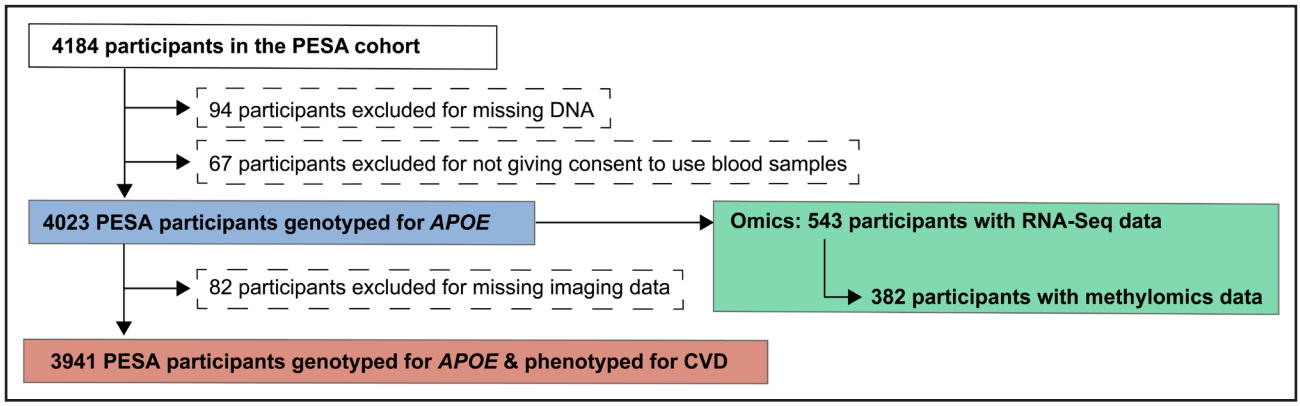

Figure 1. Study population flowchart.
Flow diagram of the selection process of the PESA (Progression of Early Subclinical Atherosclerosis) participants included in the analyses performed in this study. CVD indicates cardiovascular disease.

After excluding 82 individuals due to inadequate quality of vascular imaging or missing clinical data, a total of 3941 PESA individuals were available (Figure 1). Moreover, 54 ε2/ε4 carriers were excluded from subsequent analyses, rendering a final cohort of 3887 PESA individuals (Table). This final cohort had a mean age of 45.8±4.3 years, and 1461 (38%) participants were women. Dyslipidemia was the most prevalent CVRF (41%), followed by smoking (21%), hypertension (12%), and diabetes (2%; Table). Dyslipidemia and total, LDL- and HDL-cholesterol levels significantly differed across *APOE* groups (Table). *APOE*-ε2 carriers presented the lowest levels of total and LDL-cholesterol and ε4 carriers the highest. In turn, HDL-cholesterol levels were progressively reduced across ε2-ε3-ε4 *APOE* carriers. Interestingly, besides the lipid profile, cardiovascular risk, as measured by the 30-year-FHS and 10-year SCORE2 risk scales, was also found progressively increased across *APOE* genotypes, with ε4 carriers having the highest CVD risk and ε2 the lowest.

Cardiovascular Risk, Subclinical Atherosclerosis, and *APOE* Genotype

Since the *APOE* genotype demonstrated a significant association with CVD risk (Table), we performed a subgroup analysis on the multiterritorial extension of subclinical atherosclerosis in the PESA population according to *APOE* groups and cardiovascular risk stratification using the long-term 30-year-FHS risk score assessment and using a logistic ordinal model. ε2 carriers (ε2/ε2 and ε2/ε3) and ε4 carriers (ε3/ε4 and ε4/ε4) were compared with the reference genotype (ε3/ε3) considered to have neutral risk of CVD. The multiterritorial extent of subclinical atherosclerosis was defined as generalized, intermediate, focal, or absent, according to the PESA score, computed with the number of vascular sites affected: right and left carotids, the abdominal aorta, and the right and left iliofemoral arteries (presence of plaque by 2D-VUS), as well as the coronary vessels (CACS≥1).[22] As expected, individuals at high CVD risk (30-year-FHS≥20%) had a higher burden of multiterritorial subclinical atherosclerosis according to the PESA score, with ε4 carriers standing out with the highest proportion of intermediate and generalized disease (P=0.00041 for trend within the high-risk groups; Figure 2A). Interestingly, those individuals classified as low risk (30-year-FHS≤10%), but with generalized subclinical atherosclerosis were mostly ε4 carriers, while ε2 carriers had the highest proportion of disease-free cases and the lowest of generalized subclinical atherosclerosis at any degree of CVD risk (P=0.0015 for trend within the low-risk groups; Figure 2A). Individuals at moderate CVD risk did not show within-group differences in subclinical atherosclerosis extent across *APOE* genotypes (P=0.19). Yet,

Table. Demographics of the Study Population

	Total N=3887	*APOE* groups			*P* value
		ε2 carriers N=414	ε3/ε3 carriers (reference) N=2809	ε4 carriers N=664	
Age, y	45.8±4.3	45.8±4.1	45.8±4.3	45.8±4.2	0.84
Female	1461 (37.6)	160 (38.6)	1071 (38.1)	230 (34.6)	0.13
Body mass index, kg/m^2	26.1±3.8	26.1±3.6	26.0±3.8	26.3±3.7	0.19
Hypertension	454 (11.7)	53 (12.8)	330 (11.7)	71 (10.7)	0.29
Diabetes	75 (1.9)	7 (1.7)	56 (2)	12 (1.8)	0.97
Dyslipidemia	1594 (41.0)	122 (29.5)*	1139 (40.5)*	333 (50.1)*	1.30x10^{-11}*
Smoking	790 (20.5)	77 (18.8)	579 (20.8)	134 (20.4)	0.63
SBP, mm Hg	116.0±12.7	116.0±12.6	116.0±12.6	117.0±12.7	0.14
DBP, mm Hg	72.5±9.5	72.4±10.1	72.4±9.4	72.7±9.4	0.33
Total cholesterol, mg/dL	201.0±33.1	187.0±31.6*	201.0±32.4*	208.0±34.7*	4.79×10^{-20}*
LDL-cholesterol, mg/dL	133.0±29.5	117.0±27.0*	133.0±28.8*	141.0±30.3*	3.43×10^{-34}*
HDL-cholesterol, mg/dL	49.2±12.2	51.2±12.8*	49.2±12.2*	47.8±11.8*	1.45×10^{-5}*
Triglycerides, mg/dL	79 (59–112)	84 (59–114)	78 (59–111)	85 (62–115)	0.13
Lipoprotein (a), mg/dL	17.7 (6.8–42.6)	12.3 (5.6–31.6)	18.7 (7.5–43.9)	16.1 (5.9–41.6)	0.081
30-y FHS, %	15.2 (8.7–23.7)	12.5 (7.3–21.1)*	15.2 (8.7–23.7)*	16.7 (9.9–26.1)*	7.59×10^{-8}
SCORE2, % (n=3561)	2.2 (1.4–3.3)	2.0 (1.2–3.0)*	2.2 (1.4–3.3)*	2.3 (1.6–2.5)*	1.20×10^{-5}

Normally distributed continuous variables are indicated as mean±SD; non-normal continuous variables as median (interquartile range); and categorical variables as N (%). Statistical differences were tested for trend across ordered *APOE* groups by an extension of the nonparametric Wilcoxon rank-sum test. Sample size of each variable is the same as the total sample size (N=3887) unless otherwise indicated. Groups were defined as ε2 carriers (ε2/ε2+ε2/ε3); ε3/ε3 carriers and ε4 carriers (ε3/ε4+ε4/ε4). ε2/ε4 carriers were excluded from these analyses. DBP indicates diastolic blood pressure; FHS, Framingham Heart Study; HDL, high-density lipoprotein; LDL, low-density lipoprotein; SBP, systolic blood pressure; and SCORE2, systematic coronary risk evaluation.

*Refers to statistically significant results (P<0.05).

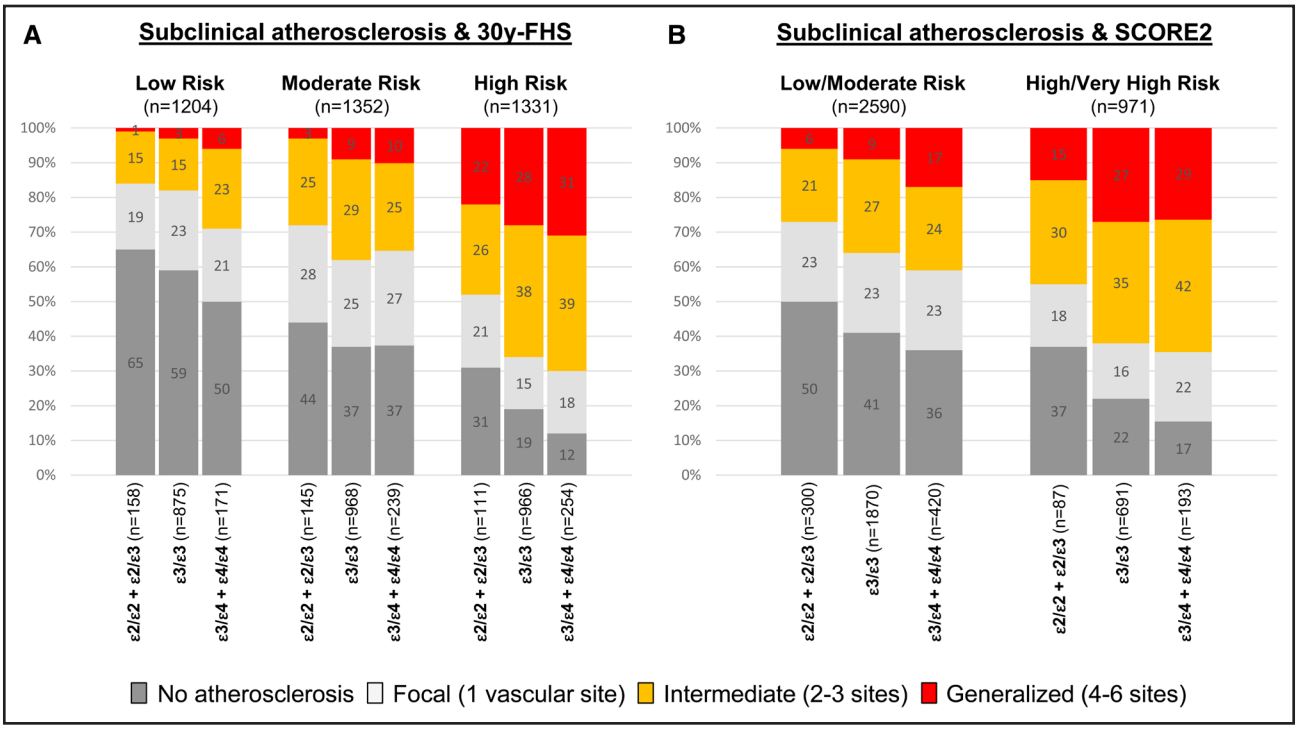

Figure 2. Interplay between *APOE* genotype, subclinical atherosclerosis, and cardiovascular risk.
Barplots showing the distribution of participants (%) across *APOE* groups, PESA (Progression of Early Subclinical Atherosclerosis) score classification, and cardiovascular disease risk according to 30-year-FHS (Framingham Heart Study; **A**) and systematic coronary risk evaluation (SCORE2; **B**). *APOE* groups were divided into ε2 carriers (ε2/ε2 and ε2/ε3), ε3/ε3 carriers, and ε4 carriers (ε3/ε4 and ε4/ε4). PESA score was defined as disease-free, focal, intermediate, and generalized disease, depending on whether there were 0, 1, 2 to 3, or 4 to 6 vascular sites affected, respectively, in the right/left carotids or femorals, aorta or coronary artery. Thirty-year-FHS groups were classified as low (≤10%), moderate (10% to 20%), and high risk (≥20%) and SCORE2 groups as low/moderate (<2.5% and age<50 or <5% and age≥50) and high/very high risk (≥2.5% and age<50 or ≥5% and age≥50). Sample sizes are indicated next to each group.

the interaction between *APOE* and the different CVD risk groups was not significant (P=0.61), suggesting that the degree to which *APOE* genotype discriminates the extent of subclinical atherosclerosis is not significantly different across CVD risk groups. Thus, the *APOE* genotype may be an independent risk factor for atherosclerosis and CVD.

The same subgroup analysis was performed with the shorter-term SCORE2 risk scale, yielding similar results (low/moderate risk: P=2.20×10^{-6}; high/very high risk: P=0.10; interaction: P=0.29; Figure 2B).

Subclinical Atherosclerosis and *APOE* Genotype

To decipher the role of *APOE* polymorphisms on subclinical atherosclerosis, we estimated the probability of having subclinical atheroma plaque based on several atherosclerosis measures of the PESA study across the different *APOE* groups by using logistic binary and ordinal regression models. Our analyses revealed that ε4 carriers had significantly higher odds of presenting atheroma plaques measured by 3D-VUS (model 1: 1.19 [1.01–1.41]; P=0.043; total plaque presence), although no significant differences were found when performing the analysis by individual vascular territories (ie, carotid or femoral; Figure 3A). Interestingly, when including the different CVRFs as covariates in the statistical model (LDL-/HDL-cholesterol, SBP/diastolic blood pressure, smoking, diabetes, and use of lipid-lowering drugs), we found that a significant association between the presence of subclinical atherosclerosis and carrying 1 or 2 copies of the ε4 allele was lost (Figure 3A, model 2). Since cholesterol levels were highly influenced by *APOE* genotype (Table), we repeated this analysis with a third model adjusted for all CVRFs but LDL-cholesterol, and no significant differences were found either (Figure 3A, model 3). Regarding the extent of multiterritorial subclinical atherosclerosis, the risk for a higher PESA score was significantly increased in ε4 carriers compared with ε3/ε3 carriers (model 1: 1.24 [95% CI, 1.07–1.42]; P=0.0035), but that significance was also lost when including CVRFs in the statistical model. However, when LDL-cholesterol was removed from the model, this analysis became significant, indicating that the increased odds ε4 carriers have at presenting multiterritorial subclinical atherosclerosis were fully dependent on their high levels of LDL-cholesterol (model 3: 1.24 [1.07–1.44]; P=0.0038; Figure 3A).

Interestingly, individuals carrying at least 1 copy of the ε2 allele consistently showed a lower probability of subclinical atherosclerosis compared with ε3/ε3 carriers,

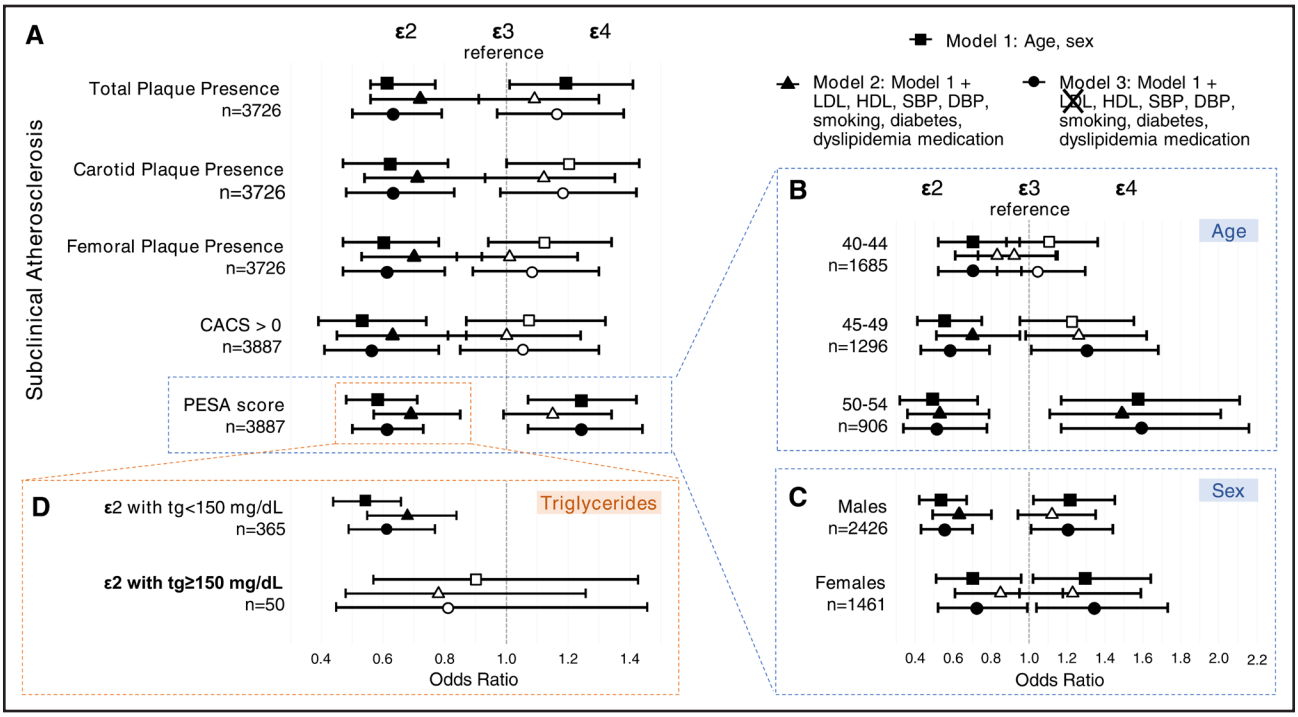

Figure 3. Effect of *APOE* alleles on subclinical atherosclerosis.
Forest plot showing the odds ratio of the presence of total, carotid and femoral plaque presence (evaluated by 3-dimensional-vascular ultrasound), coronary artery calcium score (CACS)>0 and increased PESA (Progression of Early Subclinical Atherosclerosis) score, across the different *APOE* groups, using logistic binary and ordinal regression models (**A**). Forest plots showing the odds ratio of increased PESA score across the different *APOE* groups stratified into different age ranges (40–44, 45–49, and 50–54 years; **B**) and sex (male and female; **C**). In all analyses, the likelihood of ε2 carriers (ε2/ε2 and ε2/ε3) and ε4 carriers (ε3/ε4 and ε4/ε4) of having subclinical atherosclerosis was compared with ε3/ε3 carriers (reference group, dashed gray line) with multivariate logistic binary or ordinary models, as corresponding. Forest plot showing the odds ratio of increased PESA score subdividing ε2 carriers into normal (<150 mg/dL) and high levels of triglycerides (≥150 mg/dL; tg;**D**). Model 1 was adjusted by age and sex (square symbol), model 2 by age, sex, LDL (low-density lipoprotein)- and HDL (high-density lipoprotein)-cholesterol levels, systolic blood pressure (SBP), diastolic blood pressure (DBP), smoking, diabetes, and using lipid-lowering medication (triangle symbol), and model 3 by all the variables included in model 2 except for LDL-cholesterol (circle symbol). Filled symbols represent significant values (P<0.05). Sample sizes are indicated next to each group.

having lower odds of presenting plaques when examined globally with 3D-VUS (model 1: 0.61 [95% CI, 0.56–0.77]; P=2.14×10^{-5}, total plaque presence) or by individual vascular territories (model 1: 0.62 [0.47–0.81], P=0.00043; carotid plaque presence, 0.60 [0.47–0.78]; P=9.96×10^{-5}, femoral plaque presence; Figure 3A). ε2 carriers also had a significantly lower risk of presenting CACS>0 (model 1: 0.53 [0.39–0.74], P=0.00013) and increased PESA score (model 1: 0.58 [0.48–0.71], P=3.16×10^{-8}; Figure 3A). These lower odds of having subclinical atherosclerosis remained significant after adjusting by CVRFs in all measurements, although with a slightly reduced effect size (model 2: 0.72 [95% CI, 0.56–0.91], P=0.0057; total plaque presence, 0.71 [0.54–0.93], P=0.015; carotid plaque presence, 0.70 [0.53–0.92], P=0.010; femoral plaque presence, 0.63 [0.45–0.87], P=0.0052, CACS>0; 0.69 [0.57–0.85], P=0.00029, PESA score; Figure 3A). Interestingly, we observed that the protection conferred by carrying ε2 alleles remained when LDL-cholesterol was removed from the model in all subclinical atherosclerosis measurements (model 3: 0.63 [95% CI, 0.50–0.79], P=9.53×10^{-5}; total plaque presence, 0.63 [0.48–0.83], P=0.00099; carotid plaque presence, 0.61 [0.47–0.80], P=0.00034; femoral plaque presence, 0.56 [0.41–0.78], P=0.00049, CACS>0; 0.61 [0.50–0.73], P=3.91×10^{-7}; PESA score; Figure 3A), indicating that low LDL-cholesterol levels in ε2 carriers do not fully explain the lower burden of subclinical atherosclerosis these individuals present compared with ε3/ε3 carriers.

In a post hoc analysis, subclinical atherosclerosis metrics were additionally compared between homozygous and heterozygous ε2 and ε4 *APOE* genotypes. No significant differences were found neither in demographics nor in subclinical atherosclerosis burden between ε2/ε2 and ε2/ε3, or between ε3/ε4 and ε4/ε4, probably due to the small sample size of the homozygous groups (Table S1). Additionally, no differences were found when comparing ε2/ε4 versus ε3/ε3 individuals (Table S1).

Aging and Sex Effects on Subclinical Atherosclerosis Across *APOE* Genotypes

The effect of aging on subclinical atherosclerosis in each *APOE* group was then studied by stratifying the previous

analysis into 3 age groups: 40 to 44, 45 to 49, and 50 to 54 years old. This subanalysis used the PESA score as the dependent variable, as this measure consistently differed in ε2 and ε4 carriers compared with ε3/ε3 carriers. Notably, the differences between *APOE* groups were progressively more significant with age (Figure 3B), as observed with logistic ordinal regression models. Between the ages of 40 and 49 years, ε4 carriers did not have increased odds of having generalized subclinical atherosclerosis compared with ε3/ε3 (Figure 3B). However, 50- to 54-year-old individuals carrying at least 1 ε4 allele had significantly higher odds of having subclinical atherosclerosis than ε3/ε3 carriers, independently of CVRFs and LDL-cholesterol levels (model 1: 1.57 [95% CI, 1.17–2.11], *P*=0.0030; model 2: 1.49 [1.11–2.01], *P*=0.0094; model 3: 1.59 [1.17–2.16], *P*=0.0030; Figure 3B). On the contrary, as early as 40 to 44 years of age, ε2 carriers presented a lower chance of having subclinical atherosclerosis compared with ε3/ε3, although this association was fully dependent on LDL-cholesterol levels (model 1: 0.70 [95% CI, 0.52–0.95], *P*=0.023; model 2: 0.83 [0.61–1.14], *P*=0.25; model 3: 0.70 [0.52–0.96], *P*=0.025). At later ages, between 45 and 54 years of age, the lower probability of having subclinical atherosclerosis of ε2 carriers was significant in all models, reducing to half the risk at 50 to 54 years old, regardless of LDL-cholesterol (model 3: 45–49 years: 0.58 [95% CI, 0.43–0.79], *P*=0.00059; model 3: 50–54 years: 0.51 [0.34–0.78], *P*=0.0016; Figure 3B).

When stratifying by sex, we found higher odds of increased PESA score in male ε4 carriers, which was dependent on LDL-cholesterol (model 1: 1.21 [95% CI, 1.02–1.45], *P*=0.031; model 2: 1.12 [0.94–1.35], *P*=0.21; model 3: 1.20 [1.01–1.44], *P*=0.044), and similar differences were observed in female ε4 carriers as compared with the ε3/ε3 group (model 1: 1.29 [1.02–1.64], *P*=0.037; model 2: 1.23 [0.95–1.59], *P*=0.11; model 3: 1.34 [1.04–1.73], *P*=0.022; Figure 3C). Contrarily, compared with ε3/ε3 carriers, male ε2 carriers had unequivocally lower odds of having subclinical atherosclerosis, independently of CVRFs and LDL-cholesterol levels (model 1: 0.53 [95% CI, 0.42–0.67], $P=1.92\times10^{-7}$; model 2: 0.63 [0.49–0.80], *P*=0.00019; model 3: 0.55 [0.43–0.70]; $P=1.07\times10^{-6}$), while the lower odds of subclinical atherosclerosis in female ε2 carriers were dependent on LDL-cholesterol (model 1: 0.70 [0.51–0.96], *P*=0.025; model 2: 0.85 [0.61–1.18], *P*=0.33; model 3: 0.72 [0.52–0.99], *P*=0.047; Figure 3C).

Effect of Triglyceride Levels on Subclinical Atherosclerosis in ε2-Carriers

To evaluate whether triglyceride levels could modify the atheroprotective effect of the ε2 allele, we performed a post hoc analysis further dividing ε2 carriers into normal and high levels of triglycerides (<150 or ≥150 mg/dL, respectively) and using a logistic ordinal regression model. ε2 carriers with normal triglyceride concentration presented lower chances of having subclinical atherosclerosis than ε3/ε3 individuals, independently of CVRFs and LDL-cholesterol (model 1: 0.54 [95% CI, 0.44–0.66], $P=4.70\times10^{-9}$; model 2: 0.68 [0.55–0.84], *P*=0.00039; model 3: 0.61 [0.49–0.77], $P=3.35\times10^{-5}$; Figure 3D), as previously found for the entire cohort (Figure 3A). However, ε2 carriers with high triglycerides showed nonsignificant differences with ε3/ε3 individuals for all models (model 1: 0.90 [95% CI, 0.57–1.43], *P*=0.67; model 2: 0.78 [0.48–1.26], *P*=0.31; model 3: 0.81 [0.45–1.46], *P*=0.49; Figure 3D). In a post hoc analysis, the same models were further applied to other metrics of subclinical atherosclerosis and yielded similar results for total, carotid, and femoral plaque presence (Figure S2). Interestingly, ε2-protection against coronary calcium was observed in the presence of normal or high triglyceride levels (Figure S2). Although sample sizes were small and variability was high within the high triglycerides group, these findings indicate that triglycerides may modify the protection conferred by the ε2 allele, especially in early stages of subclinical atherosclerosis development.

Differential Molecular and Cellular Pathways Across *APOE* Genotypes

Since we observed a robust protection from atherosclerosis development by the ε2 allele independently of traditional CVRFs (Figure 3), we next aimed to decipher the molecular mechanisms underlying such phenomenon. For that, we used omics data available in a subset of the PESA cohort to study the differential impact of bearing an *APOE* ε2-allele (ε2/ε2 and ε2/ε3 genotypes) on methylation and gene expression versus the other genotypes which had higher odds of presenting subclinical atherosclerosis (ε3/ε3, ε3/ε4, and ε4/ε4 genotypes). As for the entire cohort, 5 and 6 ε2/ε4 carriers were excluded from further analyses, rendering a final cohort of 377 and 537 individuals for methylomics and transcriptomics, respectively, whose demographics are shown in Table S2.

High-throughput methylomics data were used to calculate the epigenetic age of each participant and the difference with the chronological age, known as epigenetic age acceleration, was estimated based on the *Grim* epigenetic clock.[19] An unadjusted Mann-Whitney *U* test revealed a significant deceleration of the *Grim* epigenetic age in ε2 carriers compared with the other individuals (U=4773; difference in means=−1.34 [95% CI, −2.52 to −0.14]; *P*=0.028; Figure 4A). Moreover, a GSEA performed with RNA-Seq data brought to light several molecular routes differentially modulated in ε2 carriers versus the other *APOE* genotypes (Table S3). In particular, we found an enrichment of canonical pathways associated with positive regulation of type I interferon production and defense responses, suggesting an anti-inflammatory mechanism in ε2 carriers. Additionally, we found downregulation of genes involved in complement

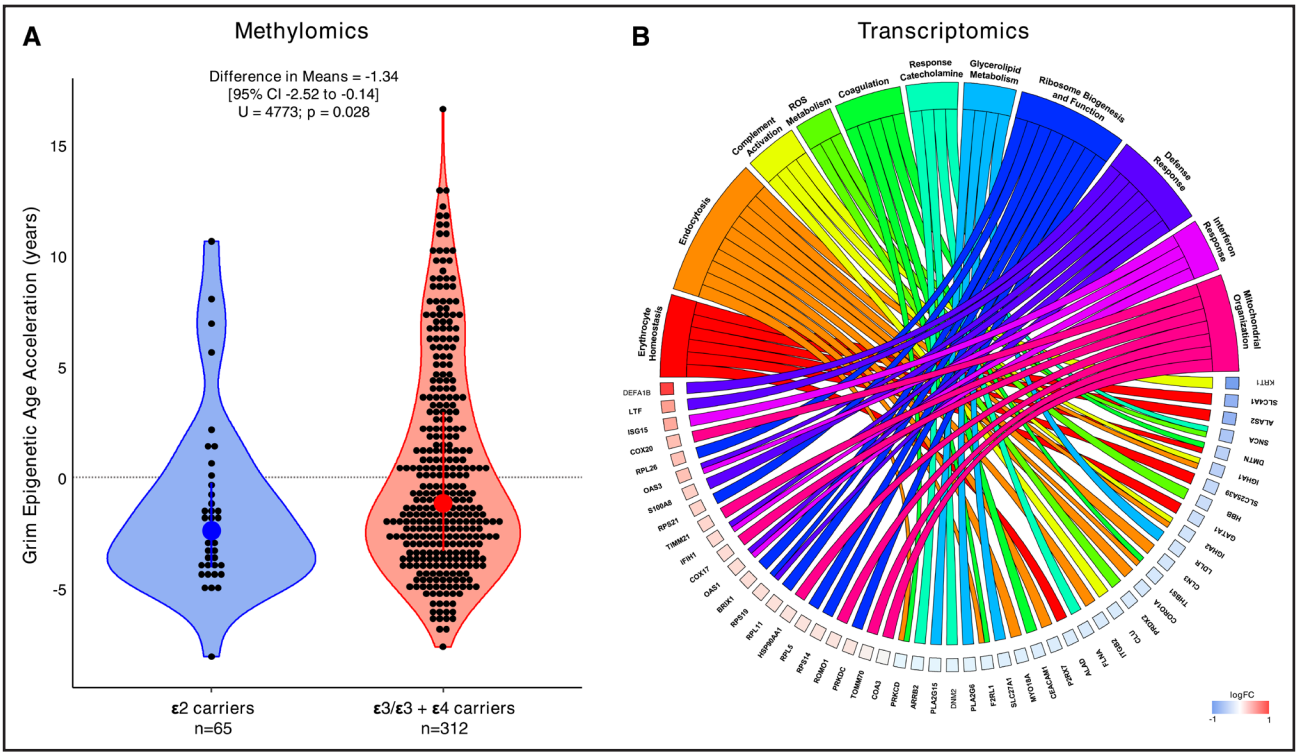

Figure 4. Omics analyses reveal differential molecular pathways in *APOE*-ε2 carriers.
Boxplot showing the *Grim* epigenetic age acceleration estimated by methylomics data for *APOE*-ε2 carriers (ε2/ε2 and ε2/ε3) vs the other genotypes (ε3/ε3, ε3/ε4, and ε4/ε4), using an unadjusted 2-sample Mann-Whitney *U* test (n=377; **A**). Chordplot of the most representative upregulated (red scale) and downregulated (blue scale) genes with summarized molecular routes enriched for a preranked gene set enrichment analysis (GSEA) upon logFC from RNA-seq data (n=537) between *APOE*-ε2 carriers vs the other genotypes (**B**). Selected categories and significant results are shown according to an FDR *q*-value<10%. Molecular routes and corresponding FDR *q*-values are shown in Table S3. ALAD indicates aminolevulinate dehydratase; ALAS2, aminolevulinate synthase 2; ARRB2, arrestin beta 2; BRIX1, biogenesis of ribosomes; CEACAM1, carcinoembryonic antigen cell adhesion molecule 1; CLN3, lysosomal/endosomal transmembrane protein battenin; CLU, clusterin; COA3, cytochrome c oxidase assembly 3; CORO1A, coronin 1A; COX17, cytochrome c oxidase copper chaperone; COX20, cytochrome c oxidase assembly factor; DEFA1B, defensin alpha 1B; DMTN, dematin actin binding protein; DNM2, dynamin 2; F2RL1, protease-activated receptor 2; FDR, False Discovery Rate; FLNA, filamin alpha; GATA1, erythroid transcription factor; HBB, hemoglobin subunit beta; HSP90AA1, heat shock protein 90 alpha member 1A; IFIH1, interferon induced with helicase C domain 1; IGHA1, immunoglobulin heavy constant alpha 1; IGHA2, immunoglobulin heavy constant alpha 2; ISG15, interferon-stimulated gene 15; ITGB2, integrin subunit beta 2; KRT1, keratin 1; LDLR, low-density lipoprotein receptor; LTF, lactotransferrin; MYO18A, myosin XVIIIA; OAS1, oligoadenylate synthetase 1; OAS3, oligoadenylate synthetase 3; P2RX7, purinergic receptor P2X 7; PLA2G15, phospholipase A2 group XV; PLA2G6, phospholipase A2 group VI; PRDX2, peroxiredoxin 2; PRKCD, protein kinase C delta; PRKDC, protein kinase DNA-activated catalytic subunit; ROMO1, reactive oxygen species modulator 1; RPL11, ribosomal protein L11; RPL26, ribosomal protein L26; RPL5, ribosomal protein L5; RPS14, ribosomal protein S14; RPS19, ribosomal protein S19; RPS21, ribosomal protein S21; SLC27A1, solute carrier family 27 member 1; SLC4A1, solute carrier family 4 member 1; SNCA, synuclein alpha; THBS1, thrombospondin 1; TIMM21, translocase of inner mitochondrial membrane 21; and TOMM70, translocase of outer mitochondrial membrane 70.

activation, regulation of coagulation and hemostasis, platelet activation, reactive oxygen species metabolism and erythrocyte homeostasis (Figure 4B).

DISCUSSION

This study shows that middle-aged asymptomatic *APOE*-ε2 carriers have lower risk of developing subclinical atherosclerosis in carotid, femoral, aorta, and coronary arteries, compared with ε3/ε3 carriers. The atheroprotective effect of the ε2 allele was independent of LDL-cholesterol, except in females and younger people (40–44 years), and was absent in individuals with high triglycerides, suggesting a window of opportunity for CVRFs to modify the genetic predisposition associated with the development of atherosclerosis and a target for prevention strategies. Moreover, using omics data, we identified several molecular and cellular pathways that may be contributing to the differential effect of APOE isoforms on atheroma development. ε2 carriers presented an enrichment of anti-inflammatory pathways and downregulation of genes involved in erythrocyte homeostasis, coagulation, and complement system—mechanisms associated with atheroma development (Graphic Abstract).

Standard CVD risk scales are useful tools to identify individuals at risk that should undergo prevention plans.[24,25] However, risk equations are not always able to accurately grasp CVD risk during early subclinical stages, especially in low-risk populations.[34] For example, most PESA individuals classified at high CVD risk indeed presented subclinical

disease, but nearly 60% of PESA participants at low risk also had subclinical atherosclerosis in at least 1 territory.[22] Hence, other factors not considered in traditional CVD risk scales may contribute to the initial asymptomatic stages of the pathology—for example, a strong genetic component is known to be involved in CVD.[9] In this study, we further characterized *APOE* as one of those factors. As expected, and consistently with other large population studies,[1,4,9] we observed that both long- and short-term risk of CVD were lower for ε2 carriers and higher for ε4 carriers in the middle-aged asymptomatic PESA cohort. However, we identified that most individuals classified as low risk for CVD but with intermediate and generalized subclinical atherosclerosis were ε4 carriers, while those disease-free were predominately ε2 carriers at any degree of CVD risk, suggesting that routine *APOE* genotyping may improve CVD risk assessment during early stages of disease progression.

Indeed, *APOE* genotype is thought to play a direct role in the development of CVD partially mediated by its impact on cholesterol levels.[11] The *APOE* gene is accountable for 7% of the interpersonal variability in total and LDL-cholesterol concentration, with compelling evidence from population studies showing that *APOE*-ε4 augments and -ε2 diminishes LDL-cholesterol serum levels.[4,32,35] Our data confirmed these reports within the PESA cohort and further revealed that ε2 carriers present higher HDL-cholesterol levels compared with ε4 carriers, in line with another country-based population study.[13] LDL-cholesterol is one of the main initiators of atherosclerosis by accumulating in the arterial wall, undergoing oxidation, and promoting the infiltration of macrophages, monocytes, and other inflammatory cells, which have an additional recognized role in atherogenesis.[36] We also found that middle-aged ε4 carriers have higher chances of presenting subclinical atherosclerosis than ε3/ε3 carriers, which was indeed mainly attributable to their higher levels of LDL-cholesterol, as reported in other middle-aged population studies.[8,13] This could not be observed in individual vascular territories, but rather when analyzing a more sensitive composite measure of the multiterritorial extent of subclinical atherosclerosis: the PESA score (ie, right/left carotids, aorta, right/left iliofemorals, and coronary arteries).[22] Increased PESA score in ε4 carriers was dependent on LDL-cholesterol levels and was mostly driven by the oldest PESA participants included in our study (ie, 50–54 years). This finding could explain why previous studies found no effect of the *APOE*-ε4 isoform on atherosclerosis in early adulthood[10] and opens a window of opportunity for ε4 carriers to possibly delay CVD through lifestyle interventions and early pharmacological treatment.

Furthermore, we identified a strong protective effect of the *APOE*-ε2 isoform on subclinical atherosclerosis development consistently throughout all the vascular territories assessed. Middle-aged ε2 carriers displayed lower odds of having subclinical atheroma plaques in the carotid and femoral arteries, coronary calcium, and generalized subclinical atherosclerosis compared with ε3/ε3 carriers. These findings agree with several studies showing that ε2 carriers have lower carotid thickness, stenosis and coronary calcium in middle- and late-life,[8–10,13,14,28,35,37] but go one step further by showing that the atheroprotective effect of the ε2 allele extends to the aorta and also to the femoral arteries, the territory with the highest burden in the PESA cohort,[22] and is mostly independent of LDL-cholesterol. The aging subanalysis expanded on these findings and revealed that generalized atherosclerosis was decreased in ε2 carriers for the entire age range of PESA participants (ie, 40–54 years). Remarkably, the strongest protective effect of *APOE*-ε2 was detected in the oldest group (ie, 50–54 years), who presented a 51% reduced risk of developing subclinical atherosclerosis compared with ε3/ε3 carriers. This healthier aging detected in ε2 carriers is in line with reports of the influence of the *APOE* genotype on longevity[38] and is further supported by our methylomics data that identified deceleration of the *Grim* epigenetic age in ε2 carriers compared with other *APOE* genotypes. The *Grim* age clock is considered a proxy of the predicted time-to-CVD and time-to-death and one of the best predictors of health and lifespan,[39] and a recent study showed acceleration of the *Grim* epigenetic age as subclinical atherosclerosis progresses in the PESA cohort.[19]

Interestingly, we found that the *APOE*-ε2 atheroprotective genetic predisposition appears to be modifiable by LDL-cholesterol levels from ages 40 to 44, suggesting that suboptimal lipid levels could hinder this protection before middle age. This LDL-dependent effect was similarly observed in females, although a confounding effect of hormonal changes and lifestyle factors may be involved.[12,28] Additionally, the *APOE*-ε2's protection was lost in individuals with elevated triglycerides. Triglyceride-rich lipoproteins are causally linked to ischemic heart disease and peripheral vascular disease[15,16] and different studies conjectured about the possible impact of high triglycerides on atheroma development in ε2 carriers.[13,40] ε2 carriers may present higher levels of circulating triglycerides, resulting from their low affinity between plasma APOE2 and LDL-cholesterol, which impairs the clearance of triglycerides from plasma.[1,3] In fact, *APOE*-ε2 homozygous are prone to develop type-III hyperlipoproteinemia, which can be triggered by additional genetic factors[3] and CVRFs.[41] Here, we found that the CVD protection conferred by the ε2 allele is triglycerides-dependent, as the risk-lowering effect present in ε2 carriers is lost when high levels of triglycerides are present. Although this result needs to be taken with caution due to the limited sample size of ε2 carriers with high triglycerides (ie, n=50), which is intrinsic to the population, this finding could have important prognostic and therapeutic implications to implement a more aggressive control of triglyceride levels in ε2 carriers. Additionally, including triglycerides in standard CVD risk equations could optimize the identification of individuals at risk.

In our study, low LDL-cholesterol in ε2 carriers did not solely explain the decreased risk of subclinical

atherosclerosis, indicating the presence of alternative pathways contributing to the ε2-atheroprotective effect. While the mechanisms responsible for this protection remain unclear in the literature, we detected using transcriptomics the enrichment of pathways involved in defense mechanisms and positive regulation of interferon production in ε2 carriers compared with other *APOE* genotypes. In steady state (ie, in the absence of an infection), type-I interferon exerts an important anti-inflammatory tolerogenic function through promoting the induction of regulatory T cells,[42,43] known to play an atheroprotective function during initial stages.[44–46] One of these upregulated genes is ISG15,[47] an interferon-induced cytokine shown to have immunomodulatory properties by modifying other proteins through a process called ISGylation.[48] Type-I interferon production also exerts anti-inflammatory effects partially by inhibiting interleukin-1 production and inflammasome activation.[49] Indeed, as one of the inflammatory mediators involved in atherosclerosis development, interleukin-1 has been a target in clinical approaches aimed at improving CVD.[50] Inflammation is recognized as an important orchestrator of atherosclerosis development[51] and a fine-tune balance between anti- and proinflammatory mediators plays a key role in the genesis of the atheroma plaque.[52] Now, we have revealed that APOE may exert isoform-specific atheroprotective/proatherogenic functions also through the modulation of inflammatory pathways. Such modulation is likely subtle but when sustained throughout life may have an important impact on atherosclerosis development.

Transcriptomics also rendered several genes downregulated in ε2 carriers involved in pathways such as erythrocyte homeostasis, coagulation, and complement activation, all intrinsically involved in atherosclerosis. Erythrocytes are putative key players in the formation of atheroma plaques, by infiltrating the intima and interacting with macrophages.[53] These cells trigger the activation of T cells and the migration of smooth muscle cells into the intima, assisted by activated platelets and other coagulation factors, that are later relevant in atherothrombogenesis as well.[54,55] Moreover, a PESA substudy has found that the activation of the complement system is a major alteration in early subclinical plaques.[56] High levels of HDL-cholesterol found in ε2 carriers could partially explain these findings considering HDL-cholesterol's potent anti-inflammatory properties[57] and reduced complement activation.[58] These differential pathways may impact the development of CVD, as well as other neurological and metabolic disorders influenced by *APOE* genotype such as Alzheimer disease, Parkinson disease, obesity, and diabetes.[59]

In sum, this study has 3 main strengths. First, the large asymptomatic middle-aged PESA cohort includes state-of-the-art imaging tools to detect multiterritorial subclinical atherosclerosis, which provides increased sensitivity over other techniques.[26] To the best of our knowledge, this is the first study reporting differences across *APOE* genotypes in generalized subclinical atherosclerosis in carotid, femoral, aorta, and coronary arteries, as most studies limit their assessments to the carotids or coronaries. As a result, we revealed that the *APOE*-ε2 protective role extends to several vascular territories. Additionally, omics data in this context identified putative underlying mechanisms behind the atheroprotective effect conferred by the ε2 allele, not explained by lipid levels or other traditional CVRFs. Lastly, our study bridges the gaps of the currently uncertain literature about age, sex, and triglyceride level interactions with *APOE* genotypes to explain subclinical atherosclerosis. This study also has several limitations, including the small number of females in the PESA cohort, especially in the omics substudy that was also enriched in individuals with subclinical atherosclerosis,[19] which could restrict the generalizability of our findings. Another limitation is that the PESA cohort is 100% White, whereas the *APOE* allele frequencies vary among ethnicities and are known to impact disease outcomes differently.[13] Also, although transcriptomics analyses identified interesting pathways altered in ε2 carriers, our studies do not prove causality of the ε2-atheroprotective effect but rather provide a potential insight into the molecular mechanisms underlying such protection that warrants further investigation.

Altogether, our results elucidate the key role of APOE isoforms in atherosclerosis development from the earliest stages and unveil that the underlying pathways responsible for the atheroprotective effect of ε2 carriers may lie on anti-inflammatory mechanisms and other pathways associated with the formation of atheroma plaques. This work also identifies a window to delay the risk associated with ε4 alleles through aggressive intervention approaches aimed at reducing CVRFs and guarantees the protection conferred by ε2 alleles by maintaining optimal levels of lipids and triglycerides, especially before middle age. Our findings also support the usefulness of implementing genetic screening in primary prevention strategies. Since the control of CVRFs has proven beneficial in modifying the APOE isoform-specific risk of CVD[60] and cognitive decline,[61,62] our study may have important therapeutic and prevention implications not only on cardiovascular health but also on cerebrovascular well being.

ARTICLE INFORMATION

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Author Contributions

Dr Toribio-Fernández was involved in conceptualization and methodology, and together with Dr Tristão-Pereira, in formal analysis, investigation and writing of the original draft. J. Carlos Silla-Castro and Dr Callejas were involved in methodology, formal analysis, and resources. I. Fernandez-Nueda performed methodology. B. Oliva was involved in data curation and formal analysis. Drs Garcia-Lunar and Garcia-Alvarez were responsible for data curation and investigation. Dr Perez-Herreras provided resources and Drs Martin, Blanco-Kelly, Ayuso, Dopazo, and Sanchez-Cabo performed investigation. Drs Lara-Pezzi, Fernandez-Ortiz, Ibanez, Cortes-Canteli, and Fuster were involved in project administration and investigation. Dr Sanchez-Cabo also performed methodology and formal analysis and provided resources. Drs Ibanez, Cortes-Canteli, and Fuster conceptualized the study and were involved in funding acquisition and were involved in writing the article. Drs Cortes-Canteli and Ibanez supervised and validated findings. All authors reviewed and approved the final version of the article.

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Disclosures

None.

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