

# **FURIN Expression in Vascular Endothelial Cells Is Modulated by a Coronary Artery Disease–Associated Genetic Variant and Influences Monocyte Transendothelial Migration**

Xu Yang, MSc; Wei Yang, MSc; David G. McVey, PhD; Guojun Zhao, PhD; Jinfu Hu, MBBS; Robin N. Poston, MD; Meixia Ren, PhD; Karin Willeit, PhD; Stefan Coassin, PhD; Johann Willeit, MD; Thomas R. Webb, PhD; Nilesh J. Samani, MD; Manuel Mayr, MD, PhD; Stefan Kiechl, MD; Shu Ye, MD, PhD

**Background**—Genome-wide association studies have shown an association between the single-nucleotide polymorphism *rs17514846* on chromosome 15q26.1 and coronary artery disease susceptibility. The underlying biological mechanism is, however, not fully understood. *rs17514846* is located in the FES Upstream Region (*FURIN*) gene, which is expressed in vascular endothelial cells (ECs). We investigated whether *rs17514846* has an influence on *FURIN* expression in ECs and whether *FURIN* affects EC behavior.

**Methods and Results**—Quantitative reverse transcription–polymerase chain reaction analysis showed that cultured vascular ECs from individuals carrying the coronary artery disease risk allele of *rs17514846* had higher *FURIN* expression than cells from noncarriers. In support, luciferase reporter analyses in ECs indicated that the risk allele had higher transcriptional activity than the nonrisk allele. Electrophoretic mobility shift assays using EC nuclear protein extracts detected a DNA-protein complex with allele-specific differential binding of a nuclear protein. Knockdown of *FURIN* in ECs reduced endothelin-1 secretion, nuclear factor- $\kappa$ B activity, vascular cell adhesion molecule-1, and MCP1 (monocyte chemoattractant protein-1) expression and monocyte-endothelial adhesion and transmigration. A population-based study showed an association of the *rs17514846* risk allele with higher circulating MCP1 levels and greater carotid intima-media thickness.

**Conclusions**—The coronary artery disease risk variant at the 15q26.1 locus modulates *FURIN* expression in vascular ECs. *FURIN* levels in ECs affect monocyte-endothelial adhesion and migration. (*J Am Heart Assoc.* 2020;9:e014333. DOI: 10.1161/JAHA.119.014333.)

**Key Words:** coronary artery disease • endothelial cells • *FURIN* • single-nucleotide polymorphism

Genome-wide association studies have identified a coronary artery disease (CAD) susceptibility locus on chromosome 15q26.1.<sup>1–3</sup> At this locus, the A allele of the lead single-nucleotide polymorphism (SNP) *rs17514846*, located in the FES Upstream Region (*FURIN*) gene, is associated with increased CAD risk.<sup>1–3</sup> The biological mechanism underlying this genetic association, however, is still not fully understood and warrants further investigation.

The *FURIN* gene encodes the subtilisin-like proprotein convertase *FURIN*, which possesses proteolytic activity

to cleave the prodomain off of, and thereby activates, certain proteins. Endothelial cells (ECs), monocytes/macrophages, and smooth muscle cells in human atherosclerotic plaques express *FURIN*.<sup>4</sup> Animal studies have demonstrated that *FURIN* promotes atherogenesis.<sup>5</sup>

Our recent study shows that *rs17514846* modulates *FURIN* expression in monocytes and that levels of *FURIN* in monocytes affect their migratory and proliferative ability,<sup>6</sup> which may partly explain the association of *rs17514846* with CAD risk.

From the Shantou University Medical College, Shantou, China (X.Y., W.Y., G.Z., J.H., S.Y.); Second Affiliated Hospital of Shantou University Medical College, Shantou, China (X.Y.); Department of Cardiovascular Sciences and National Institute for Health Research Leicester Biomedical Research Centre, University of Leicester, United Kingdom (D.G.M., T.R.W., N.J.S., S.Y.); The Sixth Affiliated Hospital of Guangzhou Medical University, Qingyuan City People's Hospital, Qingyuan, China (G.Z.); William Harvey Research Institute, Queen Mary University of London, London, United Kingdom (R.N.P.); Department of Geriatric Medicine, Fujian Provincial Hospital, Fuzhou, China (M.R.); Department of Neurology, Bern University Hospital, University of Bern, Switzerland (K.W.); Institute of Genetic Epidemiology (S.C.) and Department of Neurology (J.W., S.K.), Medical University of Innsbruck, Innsbruck, Austria; and Cardiovascular Division, King's College London, London, United Kingdom (M.M.).

**Correspondence to:** Shu Ye, MD, PhD, Shantou University Medical College, No. 22 Xinling Road, Shantou 515041, China. E-mail: s.ye@stu.edu.cn

Received August 16, 2019; accepted December 12, 2019.

© 2020 The Authors. Published on behalf of the American Heart Association, Inc., by Wiley. This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.

## Clinical Perspective

### What Is New?

- The coronary artery disease-associated single-nucleotide polymorphism *rs17514846* on chromosome 15q26.1 exerts an allele-specific effect on *FURIN* expression in vascular endothelial cells.
- The level of *FURIN* in vascular endothelial cells has an influence on monocyte-endothelial adhesion and transmigration, mediated by an effect of *FURIN* on the secretion of endothelin-1, nuclear factor- $\kappa$ B activity, and the expression of vascular cell adhesion molecule-1 and MCP1 (monocyte chemoattractant protein-1).
- Individuals carrying the *rs17514846* risk allele have higher circulating MCP1 levels and greater carotid intima-media thickness.

### What Are the Clinical Implications?

- The results of this study provide new evidence supporting a role of *FURIN* in the pathogenesis of atherosclerosis and indicating *FURIN* as a potential therapeutic target for atherosclerosis.

Because ECs in atherosclerotic plaques also express *FURIN*<sup>4</sup> and because ECs also play a vital role in atherogenesis, we sought, in the present study, to determine whether *rs17514846* has an effect on *FURIN* expression in ECs and investigate the effect of altered *FURIN* expression on EC functional behavior, with a goal to gain a more complete understanding of the biological mechanism underpinning the genetic association with CAD risk.

## Methods

The data that support the findings of this study are available from the corresponding author on reasonable request.

## Bioinformatics Analysis

Bioinformatics analysis of the genomic region containing *rs17514846* was performed. Histone modification and transcription-level data at the *rs17514846* site in human vascular ECs, reported by Encyclopedia of DNA Elements, were obtained using the University of California, Santa Cruz, Genome Browser (<https://genome.ucsc.edu/>).

## Isolation of Vascular ECs

This study had ethical approval from Queen Mary, University of London (reference: 08/H0704/140). All human tissue samples were fully anonymized before distribution to the

recipient analysis groups, as per ethical approval. Venous ECs were isolated from umbilical cords from different individuals using the collagenase digestion method, as previously described.<sup>7</sup>

## Cell Culture

Human umbilical vein ECs (HUVECs) were cultured in M199 media with supplementation, as described previously.<sup>8</sup> THP1 monocytic cells were maintained in RPMI-1640 media, supplemented with 10% (v/v) fetal bovine serum, 100 U/mL penicillin, and 100  $\mu$ g/mL streptomycin. All cells were cultured in a humidified incubator at 37°C with 5% CO<sub>2</sub>.

## Genotyping

Genomic DNA, extracted from cultured HUVECs of different individuals and from blood samples of Bruneck Study participants, was genotyped for *rs17514846* using a TaqMan SNP genotyping assay (C\_\_1244341\_10; Applied Biosystems).

## Reverse Transcription–Polymerase Chain Reaction

RNA samples, prepared from cultured HUVECs from different individuals, were reverse transcribed using random primers (Promega) and M-MLV reverse transcriptase (Promega). The resulting cDNA was subjected to real-time polymerase chain reaction for *FURIN* and the housekeeping gene *18S rRNA*, using TaqMan gene expression assays (Hs00965485\_g1 and Hs9999901\_s1; Applied Biosystems).

## Luciferase Reporter Assay

Two sets of plasmid constructs containing a firefly luciferase reporter gene were generated using the pGL4.10-promoter vector (Promega). One set of these constructs contained an inserted DNA sequence from nucleotide position chromosome 15:90873290 to chromosome 15:90873350 on the hg38 human reference sequence, with either the *A* or *C* allele at the *rs17514846* site (chromosome 15:90873320). The other set of constructs contained 3 tandemly repeated copies of the sequence from chromosome 15:90873309 to chromosome 15:90873331, with either the *A* or *C* allele at *rs17514846*. These constructs were individually combined with a *Renilla* luciferase reference plasmid (pRL-TK; Promega) and transfected into HUVECs (ATCC; CRL2480). After 48 hours, luciferase assays using the Dual-Glo Luciferase Assay System (Promega) were performed. Firefly luciferase data were normalized to *Renilla* to determine the relative activity of the cloned DNA sequences.

## Electrophoretic Mobility Shift Assay

Nuclear protein extracts from HUVECs (ATCC; CRL2480) were prepared using the NE-PER Nuclear and Cytoplasmic Extraction Reagents (ThermoFisher Scientific), according to the manufacturer's protocol. Fluorescein amidite-labeled, double-stranded DNA probes corresponding to the sequence (hg38 human reference sequence: chromosome 15:90873308 to chromosome 15:90873332) encompassing the *rs17514846* site (chromosome 15:90873320) with either the *A* or *C* at this position, were individually incubated with EC nuclear protein extracts, in the presence or absence of unlabeled *C* or *A* allele oligonucleotides and an unlabeled unrelated oligonucleotide, respectively. The mixes were subjected to 10% nondenaturing polyacrylamide gel electrophoresis, and free probes and probe-protein complexes were detected using the LightShift Chemiluminescent EMSA kit (Pierce Biotechnology; 20148). Three independent experiments were performed.

## FURIN siRNA and Transfection

HUVECs (ATCC; CRL2480) from a donor of the *A/C* genotype for *rs17514846* at passage 5 were transfected with either a FURIN siRNA (ThermoFisher Scientific; s9988) or a control siRNA (ThermoFisher Scientific; 4390843) using Lipofectamine RNAiMAX transfection reagent (Invitrogen). FURIN knockdown was verified by Western blot.

## Monocyte-Endothelial Adhesion Assay

Peripheral blood-derived primary monocytes, isolated using Dynabeads FlowComp Human CD14 kit (ThermoFisher Scientific; 11367D), were stained with CellTracker Green CMFDA Dye (ThermoFisher Scientific; C7025). Labeled monocytes ( $1 \times 10^5$  cells per well in 96-well plates) were then incubated with a monolayer of untreated and siRNA-transfected HUVECs for 1 hour, followed by washing to remove nonadhered monocytes, fixation, and fluorescence microscopy. The number of adhered monocytes was counted using ImageJ software.

## Monocyte-Endothelial Transmigration Assay

A total of  $5 \times 10^3$  HUVECs (ATCC; CRL2480) of the *A/C* genotype for *rs17514846* were seeded in 4.26-mm diameter transwells with a 5.0- $\mu$ m pore size (Corning Costar; 3388) and transfected the next day (at 60%–80% confluence) with either a FURIN siRNA or a control siRNA and cultured for 48 hours to produce a confluent monolayer. Nontransfected controls were also included in all experiments. For transmigration assays, peripheral blood-derived primary monocytes or THP1 monocytes were stained with 2.5 mmol/L CellTracker Green CMFDA Dye (ThermoFisher Scientific; C7025)

in serum-free RPMI-1640 medium for 30 minutes at 37°C. Monocytes were then washed with serum-free RPMI-1640 medium before being added to the transwell ( $1 \times 10^5$  cells per transwell in 100- $\mu$ L media). Migration was allowed to proceed for 4 hours at 37°C. After 4 hours, the transwell insert was removed and transmigration of monocytes was assessed by counting the number of cells that had migrated through the membrane.

## Western Blot Analysis

Protein lysates of HUVECs were prepared using Radio Immuno Precipitation Assay (RIPA) Lysis Buffer. Western blot analysis was performed using antibodies against FURIN (Abcam; ab183495), endothelin-1 (Abcam; ab117757), vascular cell adhesion molecule-1 (VCAM1) (Abcam; ab174279), MCP1 (monocyte chemotactic protein-1) (Abcam; ab151538), and beta-Actin (Sangon Biotech; D110001).

## Quantification of Endothelin-1 by ELISA

The levels of endothelin-1 in the supernatants of HUVEC cultures were measured using an ELISA kit (R&D Systems; QET00B) and a TECAN Infinite M200 Pro plate reader. Quantification of each sample was performed using a standard curve of known concentrations.

## Nuclear Factor $\kappa$ B p65 Transcription Factor Activity Assay

HUVEC nuclear extracts were prepared using the NE-PER Nuclear and Cytoplasmic Extraction Reagents (ThermoFisher Scientific; 78833). Nuclear factor (NF)  $\kappa$ B activity was determined using 9  $\mu$ g of nuclear extract and an NF $\kappa$ B p65 Transcription Factor Assay Kit (Abcam; ab133112), following the manufacturer's instructions. Absorbance was measured at 450 nm with a TECAN Infinite M200 Pro plate reader.

## Bruneck Study Cohort and Measurements of Plasma MCP1 Levels and Carotid Intima-Media Thickness

The Bruneck Study is a population-based study of atherosclerosis.<sup>9–12</sup> Recruitment and characteristics of the Bruneck Study cohort have been described previously.<sup>9–12</sup> The study protocol was approved by the appropriate ethics committees, and all study subjects gave written informed consent before entering the study. At the outset of the study in 1990, a sex- and age-stratified random sample of all inhabitants of Bruneck (Bolzano Province, Italy), aged 40 to 79 years (125 women and 125 men in each of the fifth to eighth decade; total  $n=1000$ ), was drawn and the individuals were invited to take part in the study. A total

of 93.6% participated, and data collection was completed for 919 subjects. The cohort was followed up in 1995, 2000, and 2005. DNA was extracted from blood samples taken from individuals (n=826) who took part in the 1995 follow-up, and used in the present study for genotyping *rs17514846*. Plasma MCP1 levels in samples taken in 2000 were determined by proximity extension assay<sup>13</sup> using Proseek Multiplex CVD I<sup>96</sup>×<sup>96</sup> reagents kits (Olink Bioscience, Sweden). Common carotid intima-media thickness was determined by Doppler ultrasound examination in 1995, as described previously.<sup>9,10</sup>

**Statistical Analyses**

Statistical analyses were undertaken using SPSS software. Reverse transcription–polymerase chain reaction C<sub>t</sub> (cycle threshold) values of *FURIN* were standardized against *18S rRNA* C<sub>t</sub> values, using the ΔC<sub>t</sub> method.<sup>14</sup> Standardized *FURIN* C<sub>t</sub> values were normalized by log transformation, and then a linear regression analysis was performed to test for an association between genotype (with *C/C*, *C/A*, and *A/A* coded as 0, 1, and 2, respectively) and log-transformed standardized *FURIN* values under an additive genetic model, whereas a *t* test was performed to ascertain an effect of genotype (with *C/C* coded as 1 and both *C/A* and *A/A* coded as 2) on log-transformed standardized *FURIN* values under an *A*-allele dominant model. The Mann-Whitney test was used to ascertain differences between *C* and *A* alleles in luciferase activity in the luciferase reporter assays and differences between control siRNA transfected cells versus *FURIN* siRNA transfected cells in the intensity of Western blot bands of *FURIN*, endothelin-1, VCAM1, and MCP1, endothelin-1 concentration in EC culture supernatants, NFκB activity, monocyte-endothelial adhesion, and monocyte-endothelial

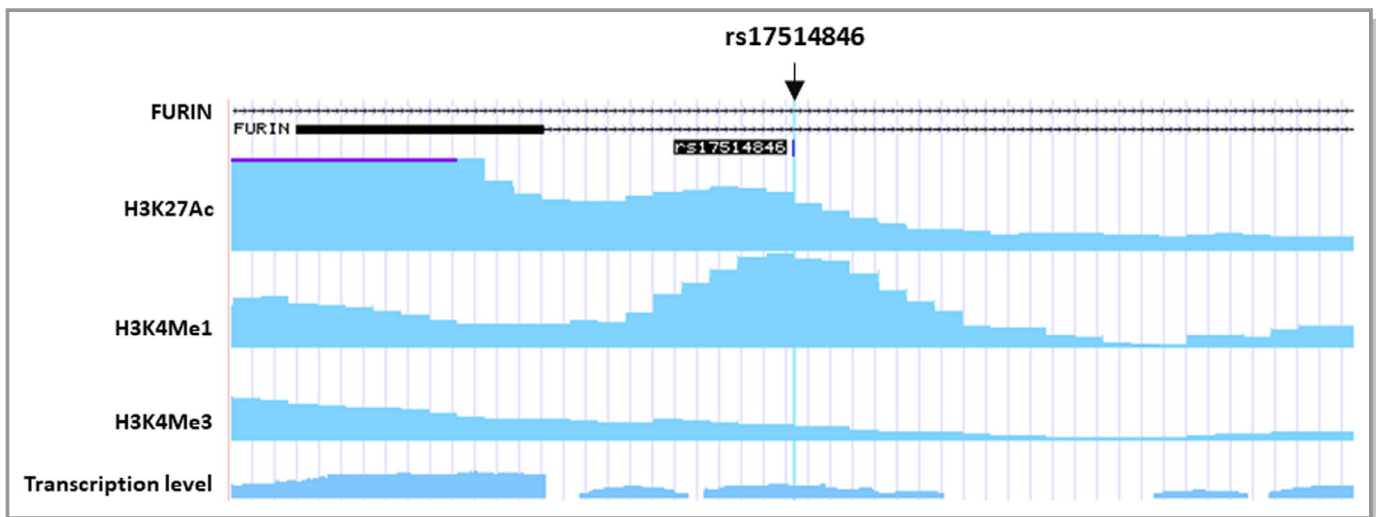
transmigration. Values of blood MCP1 levels in the Bruneck Study subjects were normalized by log transformation, and a linear regression analysis was then performed to test an association between genotype (with *C/C*, *C/A*, and *A/A* coded as 0, 1, and 2, respectively) in log-transformed MCP1 levels under an additive genetic model, whereas a general linear model analysis was performed to ascertain an effect of genotype (with *C/C* coded as 1 and both *C/A* and *A/A* coded as 2) on log-transformed MCP1 levels under an *A*-allele dominant model, with adjustment for age and sex in both additive and dominant models. A linear regression analysis was performed to test an association between genotype and carotid intima-media thickness in Bruneck Study participants with adjustment for age and sex. All *P* values were 2 sided.

**Results**

***rs17514846* Influences *FURIN* Expression in ECs**

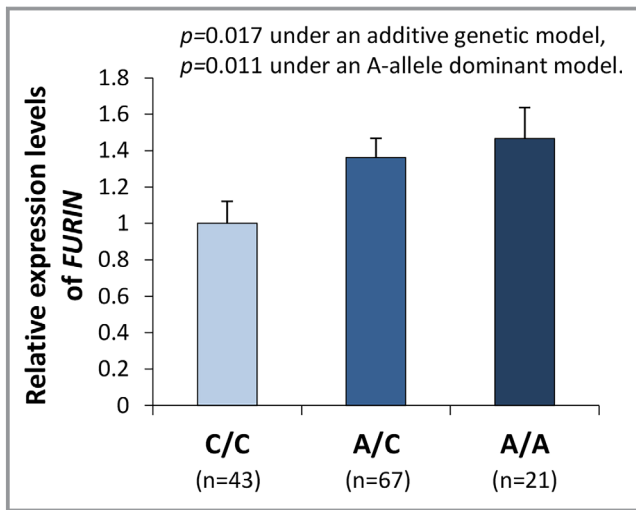
A bioinformatics analysis showed that *rs17514846* was located in a genomic region with active transcription in ECs (Figure 1). To determine if there was an allelic/genotypic effect of *rs17514846* on *FURIN* expression in ECs, we genotyped a collection of ECs from different individuals (n=131) for *rs17514846* and performed reverse transcription–polymerase chain reaction analysis to quantify *FURIN* expression levels in the cells. The analysis showed an association between *rs17514846* genotype and *FURIN* expression levels in ECs, with the *A* allele associating with higher *FURIN* expression (Figure 2).

Having detected an association between *rs17514846* genotype and *FURIN* expression in ECs, we investigated



**Figure 1.** Results of bioinformatics analysis of the genomic region containing *rs17514846*. The figure was generated from University of California, Santa Cruz, Genome Browser (<https://genome.ucsc.edu>) with data from the Encyclopedia of DNA Elements Project showing features of active transcription, including histone modification marks (H3K27Ac, H3K4Me1, and H3K4Me3) and transcription level at the *rs17514846* site, in human vascular endothelial cells.

Downloaded from <http://ahajournals.org> by on June 24, 2020



**Figure 2.** Influence of *rs17514846* genotype on *FURIN* expression in vascular endothelial cells. Primary cultures of human vascular endothelial cells from different individuals were genotyped for *rs17514846* and subjected to quantitative reverse transcription–polymerase chain reaction analysis of *FURIN*. The graph shows mean and SE values of *FURIN* expression levels in each *rs17514846* genotype group, after normalization to the *18S rRNA* gene and the C/C group.

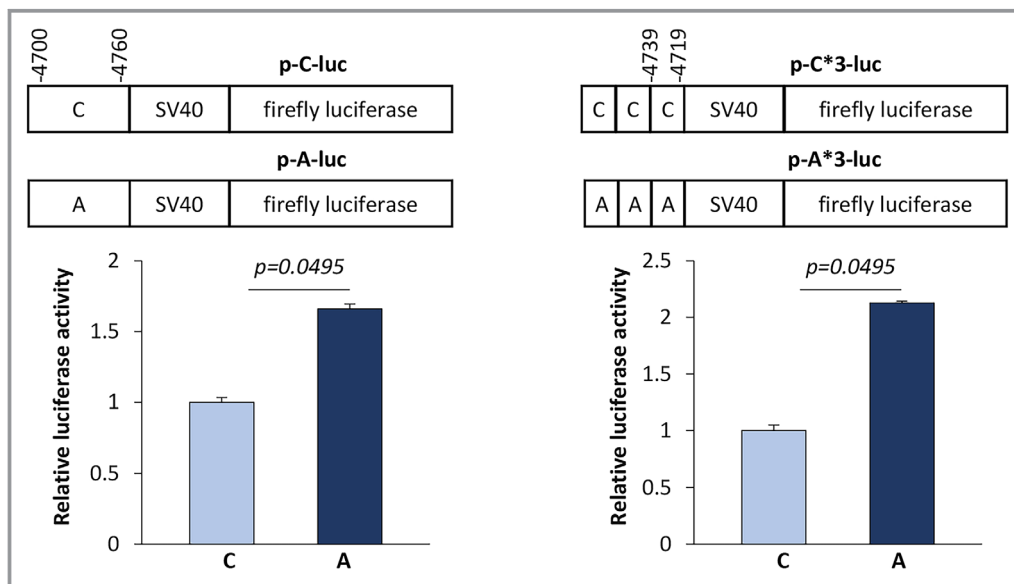
whether *rs17514846* can directly modulate gene expression using a luciferase reporter assay. Cultured ECs were transfected with a firefly luciferase reporter plasmid containing either the A or C alleles of *rs17514846*, together with a *Renilla*

luciferase plasmid to serve as a transfection efficiency control. This assay showed that cells transfected with the A allele plasmid had significantly higher firefly luciferase levels than C allele plasmid transfectants (Figure 3), suggesting that the A allele has greater strength in driving gene expression than the C allele.

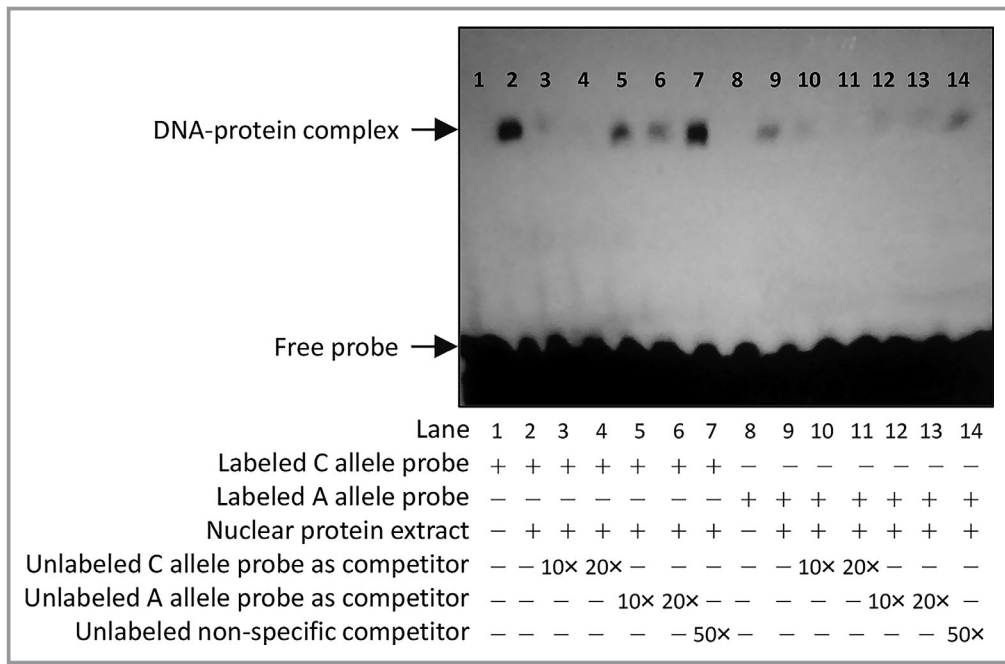
In addition, an electrophoretic mobility shift assay using EC nuclear protein extracts was performed to investigate if there was a difference between the 2 alleles of *rs17514846* in nuclear protein binding. We found preferential nuclear protein binding to the labeled C allele probe compared with the labeled A allele probe (lanes 2 and 9, respectively; Figure 4). Moreover, the DNA-protein complex was hardly detected in the presence of the unlabeled C allele probe as a competitor in 20× molar excess (lane 4; Figure 4). However, it was still readily detectable in the presence of the unlabeled A allele probe (lane 6; Figure 4). These data indicate that the C allele has greater affinity for binding of nuclear proteins compared with the A allele.

### FURIN Promotes Monocyte-Endothelial Adhesion and Transmigration

We previously demonstrated that monocyte *FURIN* levels influenced the ability of monocytes to migrate through a polycarbonate membrane (with 8-μm pores) in a transwell assay.<sup>6</sup> In the present study, we further investigated whether



**Figure 3.** Effects of *rs17514846* on gene transcription regulation. Luciferase reporter assays were performed by transfecting vascular endothelial cells with luciferase plasmids containing 60 bp of DNA encompassing *rs17514846* (left) or with luciferase plasmids containing 3, 22-bp repeats containing *rs17514846* (right). Both assays included *Renilla* luciferase as a transfection efficiency control. Columns and error bars represent mean (normalized to the C allele data) and SE values, respectively. N=3 experiments.



**Figure 4.** Allele-specific effect of *rs17514846* on nuclear protein binding. Fluorescence-labeled double-stranded 25-mer oligonucleotide probes corresponding to the sequence encompassing the *rs17514846* site, with either C or A at the *rs17514846* site, were individually incubated with or without vascular endothelial cell nuclear protein extracts, in the presence or absence of unlabeled C allele oligonucleotide, unlabeled A allele oligonucleotide, or an unrelated oligonucleotide, respectively, followed by nondenaturing PAGE and fluorescence detection of the labeled probes. Arrows indicate a DNA-protein complex and the free probe. Image shown is a representative from 3 experiments.

the level of FURIN in ECs had an effect on monocyte-endothelial adhesion and monocyte transendothelial migration. Adhesion assays and migration assays using blood-derived primary monocytes showed that siRNA-mediated FURIN knockdown in ECs (Figure 5A) inhibited monocyte adhesion to ECs (Figure 5B) and retarded monocyte transendothelial migration (Figure 5C). Migration assays using THP1 monocytes also showed a trend toward reduced transendothelial migration when FURIN was knocked down in ECs (Figure 5D).

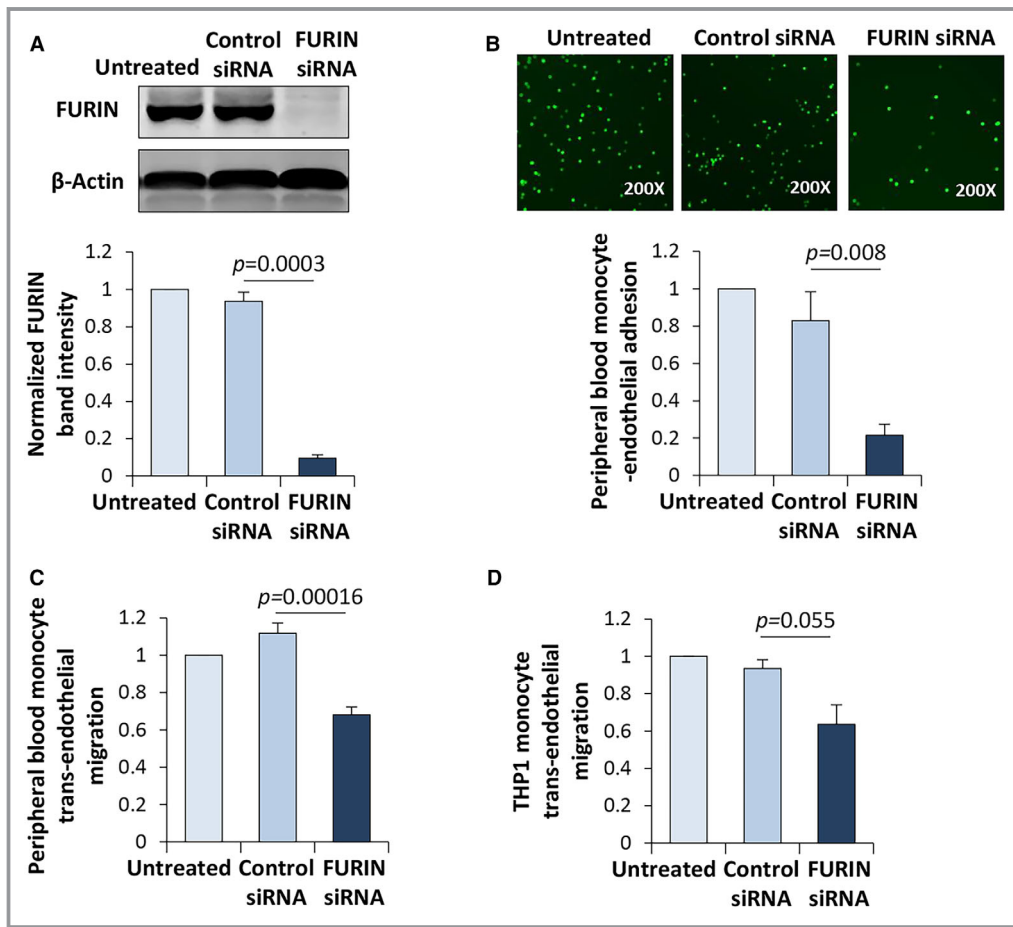
### FURIN Influences Endothelin-1 Secretion, NFκB Activity, and Expression of VCAM1 and MCP1

To investigate possible reasons for the observed inhibitory effect of FURIN knockdown on monocyte-endothelial adhesion and transmigration, we first tested whether FURIN knockdown affected endothelin-1 secretion because a previous study showed that in mice, EC-specific knockout of FURIN resulted in a substantial decrease in mature endothelin-1.<sup>15</sup> We found that knockdown of FURIN in cultured human ECs resulted in no change in cytoplasmic endothelin-1 (Figure 6A) but led to a reduction in secreted endothelin-1 (Figure 6B), suggesting a role of FURIN on endothelin-1 processing and secretion in human ECs.

Having confirmed that FURIN played a role in endothelin-1 secretion, we tested whether FURIN had an effect on the activity of the transcription factor NFκB in ECs, as previous studies showed that endothelin-1 induced NFκB activation in cancer cells.<sup>16,17</sup> Our study demonstrated that FURIN knockdown in cultured ECs resulted in reduced NFκB activity (Figure 6C), indicating a role of FURIN in NFκB activity regulation in ECs.

Because NFκB plays a critical role in the expression of VCAM1<sup>18</sup> and MCP1,<sup>19</sup> which are key mediators of monocyte-endothelial adhesion and transmigration,<sup>20</sup> we investigated whether FURIN had an effect on VCAM1 and MCP1 expression. We found that FURIN knockdown resulted in decreased expression of VCAM1 and MCP1 in ECs (Figure 6D and 6E), which is likely to account for, at least in part, the effect of FURIN on monocyte-endothelial adhesion and transmigration.

Furthermore, we found that there was an association between the A allele of SNP *rs17514846* and higher circulating MCP1 levels in a cohort of individuals who participated in a population-based study of atherosclerosis, the Bruneck Study<sup>9-12</sup> (Figure 7). We did not detect an association between *rs17514846* genotype and the level of circulating VCAM1, intercellular adhesion molecule 1, E-selection, or P-selectin in the Bruneck Study cohort.



**Figure 5.** FURIN in vascular endothelial cells affects monocyte-endothelial adhesion and transmigration. **A**, Western blot analysis of untreated and siRNA-transfected endothelial cells at 72 hours after transfection was used to confirm successful knockdown of FURIN. A representative Western blot image is shown. Band intensities were normalized to β-actin, N=9 per group. **B**, Results of monocyte-endothelial adhesion assay. Peripheral blood-derived primary monocytes were stained with CellTracker Green CMFDA and then incubated with a monolayer of untreated and siRNA-transfected endothelial cells for 1 hour, followed by washing to remove nonadhered THP1 cells, fixation, and fluorescence microscopy. The number of adhered THP1 cells was counted using ImageJ software. N=5 per group. **C** and **D**, Result of monocyte-endothelial transmigration assay. Peripheral blood-derived primary monocytes (**C**) or THP1 monocytes (**D**) were stained with CellTracker Green CMFDA and then incubated with a confluent monolayer of untreated and siRNA-transfected endothelial cells cultured on 96-well transwells. After 4 hours, the number of THP1 cells that had migrated into the lower chamber was counted. N=8 per group (**C**), and N=6 per group (**D**). **A** through **D**, Graphs show mean values normalized to untreated samples, and error bars show SE.

### rs17514846 Is Associated With Carotid Intima-Media Thickness

Further to the findings described above, we investigated if there was an association between *rs17514846* genotype and carotid intima-media thickness, a well-established biomarker of early atherosclerosis and a predictor of future cardiovascular events.<sup>21,22</sup> An analysis of individuals participating in the Bruneck Study showed an association between the A allele of *rs17514846* and greater carotid intima-media thickness (Table), which remained significant after adjusting for age and sex.

### Discussion

It has been shown that in human atherosclerotic plaques, ECs express FURIN.<sup>4</sup> A key new finding of our present study is that the CAD-associated SNP *rs17514846* at the 15q26.1 locus modulates the expression of FURIN in ECs, with the CAD risk (A) allele having higher FURIN expression levels than the nonrisk (C) allele. This complements our previously reported observation that *rs17514846* genotype affects FURIN expression in monocytes,<sup>6</sup> indicating that the CAD-associated variant exerts a regulatory effect on FURIN expression in both cell types that are known to play pivotal roles in the

**Table.** Association Between *rs17514846* Genotype and cIMT

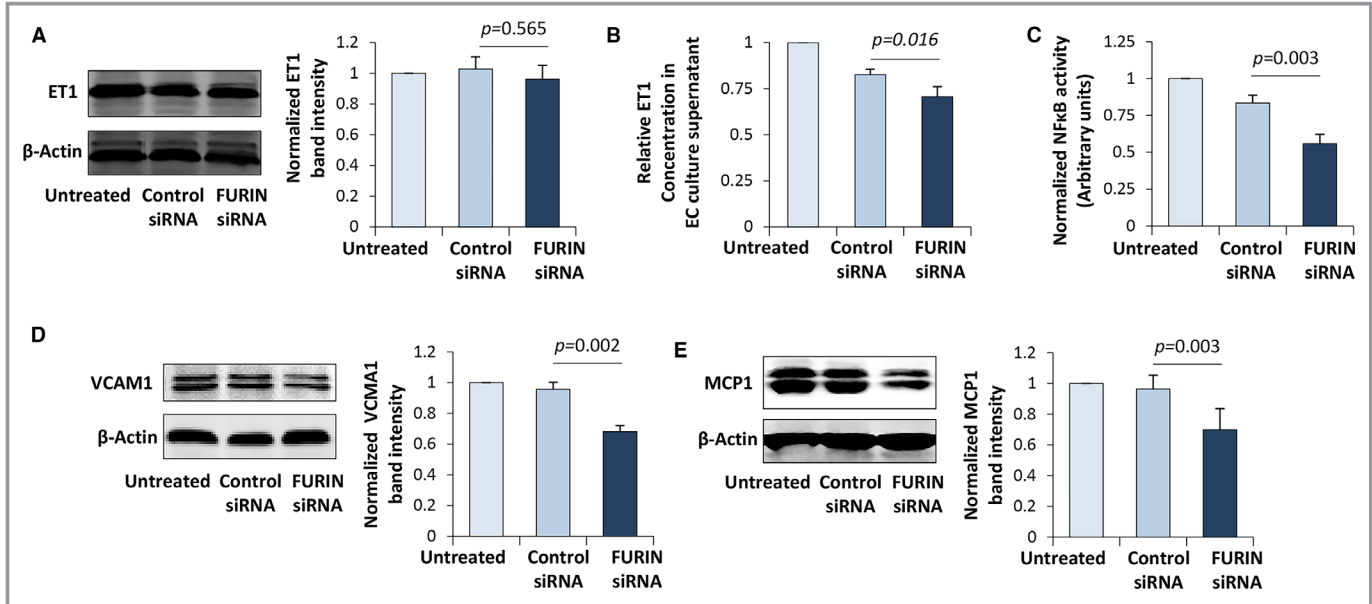
Variable	C/C (n=247)	A/C (n=401)	A/A (n=152)	P Value
cIMT, mm	0.934 (0.013)	0.943 (0.010)	0.978 (0.017)	0.046

cIMT values shown are age- and sex-adjusted means (SEs). P value shown is for an additive genetic model. cIMT indicates carotid artery intima-media thickness.

development and progression of atherosclerosis.<sup>20,23</sup> The *rs17514846* resides in intron 1 of the *FURIN* gene and is located in a genomic region with enriched binding of transcription factors, as indicated by histone 3 lysine 27 acetylation marks (Figure 1). Electrophoretic mobility shift assays, performed as part of our study, indicated that *rs17514846* has an allele-specific effect on the binding of a nuclear protein present in vascular ECs, which might possibly play a role in mediating the effect of *rs17514846* on *FURIN* expression. The findings of our study are consistent with the accumulating evidence that intronic SNPs in many genes can modulate gene expression.<sup>24,25</sup>

Second, our study demonstrates that the level of *FURIN* in ECs exerts an effect on monocyte-endothelial adhesion and transmigration. This finding is in line with, and extends on, our previous observation that *FURIN* levels in monocytes influence monocytes' migratory ability.<sup>6</sup> There is evidence

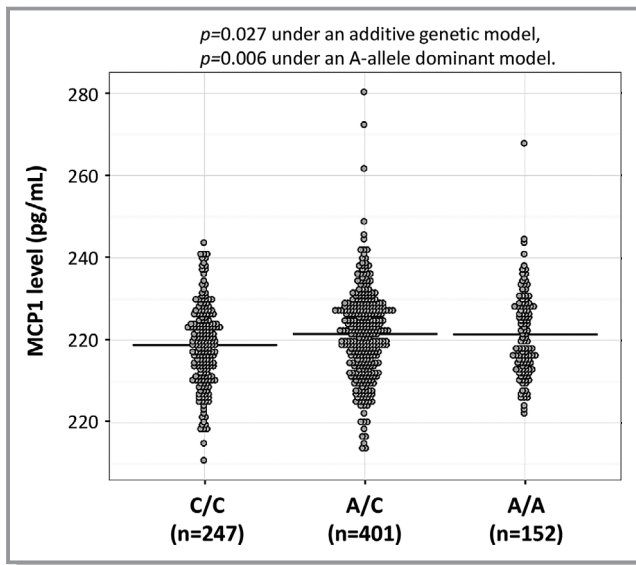
suggesting that *FURIN* may regulate endothelin-1 activity by cleaving intracellular pro-endothelin-1 and thereby facilitating the secretion of active endothelin-1.<sup>26,27</sup> In agreement, our study shows that *FURIN* knockdown resulted in reduced secretion of endothelin-1 but had no effect on cytoplasmic endothelin-1 levels. Endothelin-1 has been found to induce activation of NFκB,<sup>16,17</sup> a transcription factor that regulates the expression of genes associated with endothelial dysfunction and inflammation.<sup>28</sup> In our study, NFκB activity and the expression of VCAM1 and MCP1 in ECs were significantly reduced after *FURIN* knockdown, indicating that *FURIN* can influence endothelin-1 secretion, NFκB activity, and, in turn, VCAM1 and MCP1 expression. This can explain the finding of our study that *FURIN* knockdown inhibited monocyte-endothelial adhesion and monocyte transendothelial migration, as it is well established that VCAM1 and MCP1 play key roles in monocyte recruitment in atherogenesis.<sup>20</sup> Furthermore, consistent with the notion that an alteration in *FURIN* level can lead to a change in MCP1 expression, we found that there was an association between *rs17514846* and plasma MCP1 levels in the Bruneck Study cohort, with the CAD risk (A) allele associating with higher levels of MCP1. MCP1 levels in individuals of the A/C genotype were similar to those in individuals of the A/A genotype, suggesting that the A allele might exert a dominant effect. Interestingly, the results of



**Figure 6.** Effects of *FURIN* knockdown endothelin-1 (ET1) secretion, nuclear factor (NF) κB activity, and MCP1 (monocyte chemotactic protein-1) expression in vascular endothelial cells. *FURIN* siRNA-transfected endothelial cells at 72 hours after transfection were subjected to Western blotting or ELISA. **A**, Western blot analysis of cellular ET1. A representative Western blot image is shown. ET1 band intensities were normalized to β-actin band intensities. N=6 per group. **B**, ELISA analysis of secreted ET1 in cell culture supernatant. N=6 per group. **C**, ELISA analysis of nuclear NFκB activity. N=10 per group. **D**, Western blot analysis of vascular cell adhesion molecule-1 (VCAM1). A representative Western blot image is shown. VCAM1 band intensities were normalized to β-actin band intensities. N=9 per group. **E**, Western blot analysis of MCP1. A representative Western blot image is shown. MCP1 band intensities were normalized to β-actin band intensities. N=7 per group. **A** through **E**, Graphs show mean values normalized to untreated samples, and error bars show SE.

Downloaded from <http://ahajournals.org> by on June 24, 2020





**Figure 7.** Association between *rs17514846* genotype and circulating MCP1 (monocyte chemoattractant protein-1) levels. Blood samples from Bruneck Study participants were subjected to DNA extraction and genotyping for *rs17514846*. Plasma MCP1 levels were determined by proximity extension assay.

measurement of *FURIN* expression level in ECs from different individuals showed that the difference in *FURIN* expression level in ECs between *A/A* and *A/C* genotypes was smaller than that between the *A/C* and *C/C* genotype. Unlike the finding for MCP1, we did not detect an association between *rs17514846* genotype and the level of circulating VCAM1, intercellular adhesion molecule 1, E-selectin, or P-selectin in the Bruneck Study cohort. It is possible that this cohort did not have sufficient statistical power for these tests because of its moderate sample size.

Thus, the data we have obtained demonstrate that *rs17514846* genotype affects *FURIN* expression in ECs and that altered *FURIN* level can influence VCAM1 and MCP1 expression and monocyte-endothelial adhesion and transmigration. Furthermore, in a population-based cohort study, we observed an association between the *rs17514846* risk (*A*) allele and greater carotid intima-media thickness, a well-established biomarker of early atherosclerosis and a predictor of future cardiovascular events.<sup>21,22</sup> These findings provide an insight into the biological mechanisms linking the 15q26.1 locus and CAD risk. They also indicate *FURIN* as a potential therapeutic target for atherosclerosis. In support of this notion, a recent study showed that *FURIN* inhibition reduces atherosclerotic lesion progression in mice.<sup>5</sup>

Furthermore, the results of our study add to the accumulating evidence indicating that *FURIN* plays important roles in inflammation. In agreement with the findings reported herein and in our recent study,<sup>6</sup> Yakala et al demonstrated in a mouse model that *FURIN* inhibitor treatment resulted in

decreased expression of inflammatory and cytokine genes in vascular ECs and macrophages and reduced monocyte migration.<sup>5</sup> Furthermore, Kappert et al showed that *FURIN* promotes adipose tissue-driven macrophage chemotaxis.<sup>29</sup> On the other hand, Cordova et al reported that mice with myeloid cell-specific deletion of *FURIN* displayed an increased number of proinflammatory macrophages and increased proinflammatory gene expression in their macrophages.<sup>30</sup> It is possible that *FURIN* may play divergent roles in inflammation in different situations, and further investigations in this area would be warranted.

### Sources of Funding

We are thankful for the support of the British Heart Foundation (BHF) (PG/16/9/31995, RG/16/13/32609, RG/19/9/34655, PG/18/73/34059, and SP/19/2/344612) and the National Natural Science Foundation of China (81370202 and 81800364). This work falls under the portfolio of research conducted within the National Institute for Health Research Leicester Biomedical Research Centre. Dr Mayr is a BHF Chair Holder (CH/16/3/32406) with BHF program grant support (RG/16/14/32397). The research was also supported by the National Institute of Health Research Biomedical Research Centre, based at Guy’s and St Thomas’ National Health Service Foundation Trust and King’s College London, in partnership with King’s College Hospital and by the excellence initiative VASCage (Centre for Promoting Vascular Health in the Ageing Community), an R&D K-Centre (COMET program: Competence Centers for Excellent Technologies) funded by the Austrian Ministry for Transport, Innovation and Technology, the Austrian Ministry for Digital and Economic Affairs and the federal states Tyrol, Salzburg, and Vienna. Dr Willeit was supported by the Translational-Research-Programme grant “Tyrol Score,” funded by the “Land Tirol.”

### Disclosures

None.

### References

- CARDIoGRAMplusC4D Consortium, Deloukas P, Kanoni S, Willenborg C, Farrall M, Assimes TL, Thompson JR, Ingelsson E, Saleheen D, Erdmann J, Goldstein BA, Stirrups K, König IR, Cazier JB, Johansson A, Hall AS, Lee JY, Willer CJ, Chambers JC, Esko T, Folkersen L, Goel A, Grundberg E, Havulinna AS, Ho WK, Hopewell JC, Eriksson N, Kleber ME, Kristiansson K, Lundmark P, Lytikainen LP, Rafelt S, Shungin D, Strawbridge RJ, Thorleifsson G, Tikkanen E, Van Zuydam N, Voight BF, Waite LL, Zhang W, Ziegler A, Absher D, Altshuler D, Balmforth AJ, Barroso I, Braund PS, Burgdorf C, Claudi-Boehm S, Cox D, Dimitriou M, Do R, DIAGRAM Consortium, CARDIOGENICS Consortium, Doney AS, El Mokhtari N, Eriksson P, Fischer K, Fontanillas P, Franco-Cereceda A, Gigante B, Groop L, Gustafsson S, Hager J, Hallmans G, Han BG, Hunt SE, Kang HM, Illig T, Kessler T, Knowles JW, Kolovou G, Kuusisto J, Langenberg C, Langford C, Leander K, Lokki ML, Lundmark A, McCarthy MI, Meisinger C, Melander O, Mihailov E, Maoouche S, Morris AD, Muller-Nurasyid M, Mu TC, Nikus K, Peden JF, Rayner NW, Rasheed A, Rosinger S, Rubin D, Rumpf MP,

Schafer A, Sivananthan M, Song C, Stewart AF, Tan ST, Thorgerirsson G, van der Schoot CE, Wagner PJ; Wellcome Trust Case Control Consortium, Wells GA, Wild PS, Yang TP, Amouyel P, Arveiler D, Basart H, Boehnke M, Boerwinkle E, Brambilla P, Cambien F, Cupples AL, de Faire U, Dehghan A, Diemert P, Epstein SE, Evans A, Ferrario MM, Ferrieres J, Gauguier D, Go AS, Goodall AH, Gudnason V, Hazen SL, Holm H, Iribarren C, Jang Y, Kahonen M, Kee F, Kim HS, Klopp N, Koenig W, Kratzer W, Kuulasmaa K, Laakso M, Laaksonen R, Lee JY, Lind L, Ouwehand WH, Parish S, Park JE, Pedersen NL, Peters A, Quertermous T, Rader DJ, Salomaa V, Schadt E, Shah SH, Sinisalo J, Stark K, Stefansson K, Tregouet DA, Virtamo J, Wallentin L, Wareham N, Zimmermann ME, Nieminen MS, Hengstenberg C, Sandhu MS, Pastinen T, Syvanen AC, Hovingh GK, Dedoussis G, Franks PW, Lehtimäki T, Metspalu A, Zalloua PA, Siegbahn A, Schreiber S, Ripatti S, Blankenberg SS, Perola M, Clarke R, Boehm BO, O'Donnell C, Reilly MP, Marz W, Collins R, Kathiresan S, Hamsten A, Kooner JS, Thorsteinsdottir U, Danesh J, Palmer CN, Roberts R, Watkins H, Schunkert H, Samani NJ. Large-scale association analysis identifies new risk loci for coronary artery disease. *Nat Genet.* 2013;45:25–33.

2. Nikpay M, Goel A, Won HH, Hall LM, Willenborg C, Kanoni S, Saleheen D, Kyriakou T, Nelson CP, Hopewell JC, Webb TR, Zeng L, Dehghan A, Alver M, Armasu SM, Auro K, Bjorndal A, Chasman DI, Chen S, Ford I, Franceschini N, Gieger C, Grace C, Gustafsson S, Huang J, Hwang SJ, Kim YK, Kleber ME, Lau KW, Lu X, Lu Y, Lytykainen LP, Mihailov E, Morrison AC, Pervjakova N, Qu L, Rose LM, Salfati E, Saxena R, Scholz M, Smith AV, Tikkanen E, Uitterlinden A, Yang X, Zhang W, Zhao W, de Andrade M, de Vries PS, van Zuydam NR, Anand SS, Bertram L, Beutner F, Dedoussis G, Frossard P, Gauguier D, Goodall AH, Gottesman O, Haber M, Han BG, Huang J, Jalilzadeh S, Kessler T, König IR, Lannfelt L, Lieb W, Lind L, Lindgren CM, Lokki ML, Magnusson PK, Mallick NH, Mehra N, Meitinger T, Memon FU, Morris AP, Nieminen MS, Pedersen NL, Peters A, Rallidis LS, Rasheed A, Samuel M, Shah SH, Sinisalo J, Stirrups KE, Trompet S, Wang L, Zaman KS, Ardisino D, Boerwinkle E, Borecki IB, Bottinger EP, Buring JE, Chambers JC, Collins R, Cupples LA, Danesh J, Demuth I, Elosua R, Epstein SE, Esko T, Feitosa MF, Franco OH, Franzosi MG, Granger CB, Gu D, Gudnason V, Hall AS, Hamsten A, Harris TB, Hazen SL, Hengstenberg C, Hofman A, Ingelsson E, Iribarren C, Jukema JW, Karhunen PJ, Kim BJ, Kooner JS, Kullo IJ, Lehtimäki T, Loos RJ, Melander O, Metspalu A, Marz W, Palmer CN, Perola M, Quertermous T, Rader DJ, Ridker PM, Ripatti S, Roberts R, Salomaa V, Sanghera DK, Schwartz SM, Seedorf U, Stewart AF, Stott DJ, Thiery J, Zalloua PA, O'Donnell CJ, Reilly MP, Assimes TL, Thompson JR, Erdmann J, Clarke R, Watkins H, Kathiresan S, McPherson R, Deloukas P, Schunkert H, Samani NJ, Farrall M; CARDIoGRAMplusC4D Consortium. A comprehensive 1,000 genomes-based genome-wide association meta-analysis of coronary artery disease. *Nat Genet.* 2015;47:1121–1130.

3. van der Harst P, Verweij N. Identification of 64 novel genetic loci provides an expanded view on the genetic architecture of coronary artery disease. *Circ Res.* 2018;122:433–443.

4. Stawowy P, Kallisch H, Borges Pereira Stawowy N, Stibenz D, Veinot JP, Grafe M, Seidah NG, Chretien M, Fleck E, Graf K. Immunohistochemical localization of subtilisin/kexin-like proprotein convertases in human atherosclerosis. *Virchows Arch.* 2005;446:351–359.

5. Yakala GK, Cabrera-Fuentes HA, Crespo-Avilan GE, Rattanasopa C, Burlacu A, George BL, Anand K, Mayan DC, Corliano M, Hernandez-Resendiz S, Wu Z, Schwerk AMK, Tan ALJ, Trigueros-Motos L, Chevre R, Chua T, Kleemann R, Liehn EA, Hausenloy DJ, Ghosh S, Singaraja RR. FURIN inhibition reduces vascular remodeling and atherosclerotic lesion progression in mice. *Arterioscler Thromb Vasc Biol.* 2019;39:387–401.

6. Zhao G, Yang W, Wu J, Chen B, Yang X, Chen J, McVey DG, Andreadi C, Gong P, Webb TR, Samani NJ, Ye S. Influence of a coronary artery disease-associated genetic variant on FURIN expression and effect of furin on macrophage behavior. *Arterioscler Thromb Vasc Biol.* 2018;38:1837–1844.

7. Baudin B, Bruneel A, Bosselut N, Vaubourdonne M. A protocol for isolation and culture of human umbilical vein endothelial cells. *Nat Protoc.* 2007;2:481–485.

8. Zhang R, Witkowska K, Afonso Guerra-Assuncao J, Ren M, Ng FL, Mauro C, Tucker AT, Caulfield MJ, Ye S. A blood pressure-associated variant of the SLC39A8 gene influences cellular cadmium accumulation and toxicity. *Hum Mol Genet.* 2016;25:4117–4126.

9. Kiechl S, Willeit J. The natural course of atherosclerosis, part I: incidence and progression. *Arterioscler Thromb Vasc Biol.* 1999;19:1484–1490.

10. Kiechl S, Willeit J; Bruneck Study Group. The natural course of atherosclerosis, part II: vascular remodeling. *Arterioscler Thromb Vasc Biol.* 1999;19:1491–1498.

11. Kiechl S, Lorenz E, Reindl M, Wiedermann CJ, Oberhollenzer F, Bonora E, Willeit J, Schwartz DA. Toll-like receptor 4 polymorphisms and atherogenesis. *N Engl J Med.* 2002;347:185–192.

12. Ye S, Willeit J, Kronenberg F, Xu Q, Kiechl S. Association of genetic variation on chromosome 9p21 with susceptibility and progression of atherosclerosis: a population-based, prospective study. *J Am Coll Cardiol.* 2008;52:378–384.

13. Assarsson E, Lundberg M, Holmquist G, Björkstén J, Thorsen SB, Ekman D, Eriksson A, Rennel Dickens E, Ohlsson S, Edfeldt G, Andersson AC, Lindstedt P, Stenvang J, Gullberg M, Fredriksson S. Homogenous 96-plex PEA immunoassay exhibiting high sensitivity, specificity, and excellent scalability. *PLoS One.* 2014;9:e95192.

14. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2<sup>-ΔΔC<sub>T</sub></sup> Method. *Methods.* 2001;25:402–408.

15. Kim W, Essalmani R, Szumska D, Creemers JW, Roebroek AJ, D'Orleans-Juste P, Bhattacharya S, Seidah NG, Prat A. Loss of endothelial furin leads to cardiac malformation and early postnatal death. *Mol Cell Biol.* 2012;32:3382–3391.

16. Cianfrocca R, Tocci P, Semprucci E, Spinella F, Di Castro V, Bagnato A, Rosano L. Beta-arrestin 1 is required for endothelin-1-induced NF-kappaB activation in ovarian cancer cells. *Life Sci.* 2014;118:179–184.

17. Felix M, Guyot MC, Isler M, Turcotte RE, Doyon J, Khatib AM, Leclerc S, Moreau A, Moldovan F. Endothelin-1 (Et-1) promotes MMP-2 and MMP-9 induction involving the transcription factor NF-kappaB in human osteosarcoma. *Clin Sci (Lond).* 2006;110:645–654.

18. Shu HB, Agranoff AB, Nabel EG, Leung K, Duckett CS, Neish AS, Collins T, Nabel GJ. Differential regulation of vascular cell adhesion molecule 1 gene expression by specific NF-kappa B subunits in endothelial and epithelial cells. *Mol Cell Biol.* 1993;13:6283–6289.

19. Ueda A, Okuda K, Ohno S, Shirai A, Igarashi T, Matsunaga K, Fukushima J, Kawamoto S, Ishigatsubo Y, Okubo T. NF-kappa B and Sp1 regulate transcription of the human monocyte chemoattractant protein-1 gene. *J Immunol.* 1994;153:2052–2063.

20. Libby P. Inflammation in atherosclerosis. *Nature.* 2002;420:868–874.

21. Lorenz MW, Markus HS, Bots ML, Rosvall M, Sitzer M. Prediction of clinical cardiovascular events with carotid intima-media thickness: a systematic review and meta-analysis. *Circulation.* 2007;115:459–467.

22. O'Leary DH, Polak JF, Kronmal RA, Manolio TA, Burke GL, Wolfson SK Jr; Cardiovascular Health Study Collaborative Research Group. Carotid-artery intima and media thickness as a risk factor for myocardial infarction and stroke in older adults. *N Engl J Med.* 1999;340:14–22.

23. Ross R. The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature.* 1993;362:801–809.

24. Cooper DN. Functional intronic polymorphisms: buried treasure awaiting discovery within our genes. *Hum Genomics.* 2010;4:284–288.

25. Yang W, Ng FL, Chan K, Pu X, Poston RN, Ren M, An W, Zhang R, Wu J, Yan S, Situ H, He X, Chen Y, Tan X, Xiao Q, Tucker AT, Caulfield MJ, Ye S. Coronary-heart-disease-associated genetic variant at the COL4A1/COL4A2 locus affects COL4A1/COL4A2 expression, vascular cell survival, atherosclerotic plaque stability and risk of myocardial infarction. *PLoS Genet.* 2016;12:e1006127.

26. Denault JB, Claigne A, D'Orleans-Juste P, Sawamura T, Kido T, Masaki T, Leduc R. Processing of proendothelin-1 by human furin convertase. *FEBS Lett.* 1995;362:276–280.

27. Blais V, Fugere M, Denault JB, Klarskov K, Day R, Leduc R. Processing of proendothelin-1 by members of the subtilisin-like pro-protein convertase family. *FEBS Lett.* 2002;524:43–48.

28. Collins T, Cybulsky MI. NF-kappaB: pivotal mediator or innocent bystander in atherogenesis? *J Clin Invest.* 2001;107:255–264.

29. Kappert K, Meyborg H, Fritzsche J, Urban D, Kruger J, Wellenhofer E, Kintscher U, Fleck E, Stawowy P. Proprotein convertase subtilisin/kexin type 3 promotes adipose tissue-driven macrophage chemotaxis and is increased in obesity. *PLoS One.* 2013;8:e70542.

30. Cordova ZM, Gronholm A, Kytola V, Taverniti V, Hamalainen S, Aittomäki S, Niininen W, Juntila I, Ylipää A, Nykter M, Pesu M. Myeloid cell expressed proprotein convertase FURIN attenuates inflammation. *Oncotarget.* 2016;7:54392–54404.