The molecular basis of early onset cardiovascular disease
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Citation for published version (APA):

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KERATOCAN; A MATRIX PROTEIN INVOLVED IN Atherosclerosis


*Equally contributed

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Submitted
ABSTRACT

Background
Heritable factors account for 30%–60% of the inter-individual variation in the risk of cardiovascular disease (CVD) and only a minute fraction of the genes underlying this inheritance has been identified. We set out to unravel the molecular pathology underlying premature CVD in a large kindred with autosomal dominant expression of atherosclerotic vascular disease.

Methods and Results
We studied a large family comprising 4 generations, of which 11 family members suffered from early onset CVD. Classical linkage analysis revealed that a 4.4 Mb interval on chromosome 12 was linked to the phenotype with a parametric LOD-score of 3.31. Subsequent capture and sequencing of this region resulted in the identification of one non-synonymous variant in Kera (NM_007035.3: c.920C>G; p.Ser307Cys), encoding for Keratocan, an extracellular matrix protein. This specific variant was not present in 9000 healthy control individuals nor in 1400 patients suffering from premature CVD. Upon sequencing the gene, we did not identify mutations in an additional 300 premature CVD cases. By immunochemistry we showed that Keratocan was not present in healthy arterial walls, but was highly expressed in the lipid rich areas of early atherosclerotic lesions and end stage plaques. Furthermore, in a mouse model where atherosclerosis was induced by cuff placement in the vessel wall, Keratocan expression was shown to be linearly associated with plaque size ($r^2 = 0.7$, $p < 0.001$).

Conclusion
By studying a large kindred with early onset CVD, we identified a rare variant in Kera, encoding for Keratocan. The potential biological relevance is exemplified by its presence in atherosclerotic plaques and correlation with atherosclerosis progression in humans and mice.
INTRODUCTION

Cardiovascular disease (CVD) remains the major cause of morbidity and mortality worldwide. Twin studies indicate that the heritability for CVD varies between 30-60%\textsuperscript{1}. The relevance of heritability is exemplified by the fact that apart from the classical risk factors, a positive family history for premature CVD in a first degree relative is independently associated with future events\textsuperscript{2}. This heritability is thought to be conferred through a combination of common and rare genetic variants with variable effects on disease expression \textsuperscript{3}. Approximately 10% of the inter-individual variation in CVD prevalence is explained by a combination of common genetic variants in twenty-five loci identified by Genome Wide Association (GWA) studies\textsuperscript{1}. Rare variants with a large effect on CVD have so far been identified by studying families with a Mendelian inheritance pattern for a given CVD risk factor. The best example of a monogenic disorder that underlies CVD is Familial Hypercholesterolemia (FH), where mutations in the genes encoding for the LDL-receptor, apolipoprotein B (ApoB) or Proprotein convertase subtilisin/kexin type 9 (PCSK-9), cause elevated LDL-c levels and early onset CVD\textsuperscript{4}. However, FH explains only a small fraction of early onset CVD in families.

Our aim was to identify the molecular defect in a large family with an autosomal dominant form of premature CVD, in the absence of established risk factors or FH, and to elucidate the role of this defect in the pathogenesis of atherosclerosis.

SUBJECTS AND METHODS

Recruitment of the study family with early onset CVD

The study was approved by the Institutional Review Board of the Academic Medical Center (AMC) of the University of Amsterdam and all participants provided written informed consent. The index case (III:8) of this Caucasian family suffered from an acute myocardial infarction (AMI) at the age of 49 years. The only CVD risk factor discerned was a moderately elevated BMI of 28 kg/m\textsuperscript{2}. Lipids and lipoproteins were all within the normal range. (Low-density lipoprotein cholesterol (LDL-c) 4.0 mmol/L, High-density lipoprotein cholesterol (HDL-c) 1.0 mmol/L, Triglycerides (TG) 1.4 mmol/L, Total cholesterol (TC) 5.7 mmol/L, and Lp(a) 40 mg/L). The extended kindred showed an autosomal dominant inheritance pattern of early onset CVD, including both cardiac and cerebral atherosclerotic ischemic events (Fig. 1 and supplementary information Table 1). From all family members who gave informed consent, blood was collected for measurements of lipid profiles and glucose levels and DNA isolation\textsuperscript{5, 6}. FH was ruled out by clinical assessment and by sequencing exon-intron boundaries of the \textit{LDLR, PCSK9} and \textit{APOB} genes.
genes and excluding known abnormal copy number variations in these genes using Multiplex Ligation-dependent Probe Amplification (MLPA) technique in the index case and in IV:27,8

Figure 1. Co-segregation of the KERA variant in a large family with early onset CVD

Squares represent males. Circles represent females. Half-filled symbols represent cases with premature cardiovascular disease (CVD). The arrow indicates the index case. For each individual the first number is an identifier within their generation, the second line is the type of CVD and the last line is the age of onset of disease in years. In the unaffected individuals the age at inclusion in years is presented. DNA of all living 12 individuals was included in genetic analysis. The Keratocan variant co-segregated perfectly with the CVD phenotype.

AMI; Acute Myocardial Infarction, AP; Angina Pectoris, PTCA; Percutaneous Transluminal Coronary Angioplasty, CABG; Coronary Artery Bypass Graft, CVA; Cerebrovascular Accident and TIA; Transient Ischemic Attack.

Genetic linkage analysis
Family members who suffered from early onset CVD, defined as a clinical CVD event ≤ 56 years for male and ≤ 65 years for female individuals (see supplementary appendix methods: CVD Definitions) were considered “affected” and family members beyond this age without any signs or complaints of CVD were considered “unaffected”. We classified III-4 as being affected, despite the fact that he suffered from CVD at the age of 58 years, because he should be the pivotal factor in passing through any causal defect to his offspring (IV-1 and IV-2), who were considered affected. The mother of IV-1 and IV-2 (III-5) did not suffer from CVD, and died from the complications of a pancreatitis at the age of 67 years.

We genotyped a total of 12 family members, of whom 9 were affected and 3 unaffected. Genotyping was performed using Human CytoSNP-12 DNA analysis BeadChip
kits according to manufacturer’s protocols (Illumina, San Diego, USA). Multipoint parametric linkage analysis assuming a fully penetrant autosomal dominant model and minor allele frequency of 0.001 was conducted using the Allegro program\(^9\). A LOD score of 3.0 or above was considered significant.

A Nimblegen (Madison, USA) custom sequence capture array comprising 395K probes was designed to enrich for the target region. Any identified non-synonymous rare variant present in dbSNP, and the available data from the 1000 genomes pilot project was excluded (see supplementary information methods: genetics)\(^10\). Co-segregation was confirmed by Sanger sequencing (see supplementary information methods: genetics). This resulted in the identification of the variant (NM_007035.3: c.920C>G; p.Ser307Cys) in KERA, a gene encoding for Keratocan.

The frequency of this novel variant was assessed in 1400 early onset CVD patients (AMC-PAS cohort and Boston Cohort), and in 9000 healthy controls (1000 Sanquin and 8000 Cambridge Bioresource cohorts; (see supplementary information methods: cohorts). The coding sequence and exon-intron boundaries of KERA were sequenced in 300 patients with premature CVD in an attempt to further substantiate the role of KERA in atherosclerosis (see supplementary information methods: genetics).

**KERA and Cornea**

Variants in KERA have been described to cause autosomal recessive Cornea Plana 2 (CP2). CP is a rare disorder characterized by an abnormal large corneal radius curvature, resulting in hypermetropia, astigmatism and poor acuity in most cases. Two carriers of the Kera mutation (IV-2 and IV-2) underwent split lamp examination to assess whether stigmata of CP2 were present. To address whether (premature) CVD is present in CP patients, through the Dutch Cornea Physicians Network, we contacted Dutch CP patients and their first degree family members and their physicians.

**Keratocan expression in human arterial vessel wall**

For immunohistochemical studies specimens of atherosclerotic and normal arterial wall were collected from 9 patients at autopsy (coronary artery sections: n=10; aorta: n=5) and vascular surgery (carotid endarterectomy specimens: n= 8; mammary arteries sections: n=6). Mammary arteries were included, since they are known to be relatively resistant to development of atherosclerosis\(^11\). All specimens were routinely formalin fixed and paraffin embedded. With the use of haematoxylin and eosin stains all arterial segments were histomorphology classified onto 4 groups as: Grade 0: no lesion or intimal thickening, corresponding with macroscopically normal arterial wall (AHA classification type 1 n=5); Grade 1: fatty streaks (AHA type II, n=5). Grade 2: Lipid rich plaques (AHA type IV, Va), and fibrous plaques (AHA type Vb,c) in total n=10, Grade 3: thrombosed plaques (AHA type VI, n=3).
For immunohistochemistry staining of Keratocan, 5 \( \mu \)m thick tissue sections were dewaxed in xylene and alcohol, and subsequently endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in methanol. The slides were subjected to a heat-induced antigen retrieval procedure with citrate pH 6.0 (20 min, 98\(^\circ\)C) in the PreTreatment Module (PTModule, Thermo Fisher Scientific/Labvision, Fremont, CA, USA). After a general protein blocking step (Ultra V Block, ThermoFisher Scientific) the slides were incubated with anti-Keratocan 1:1000 (rabbit IgG, Sc6694 Santa Cruz Biotechnology, Santa Cruz, California, USA) overnight at 4\(^\circ\)C, followed by anti-rabbit IgG/HRP-labeled polymer (ImmunoLogic, Duiven, The Netherlands). Horse Radish Peroxide (HRP) activity was demonstrated by brown staining with Bright DAB+ (ImmunoLogic, Duiven, The Netherlands). Slides were counterstained with hematoxylin, fully dried with a hot plate and coverslipped with VectaMount (Vector Laboratories, Burlingame, California, USA).

Immunodouble staining experiments were performed to address the cell specificity of KERA expression. Keratocan antibody was used in combination with a specific antibody for smooth muscle cells (anti SMA, monoclonal 1A4, DAKO, Glostrup, Denmark), macrophages (antiCD68, monoclonal PG-M1, DAKO), and endothelial cells (anti CD34, monoclonal QBend10, ThermoFisher Scientific).

To study the relationship between Keratocan and the presence of CXCL1 in the vessel wall anti-CXCL1; 1:10.000 (rabbit anti-human polyclonal antibody, LS-B2843, LifeSpan Biosciences, Seattle, Washington, USA) was used. Triple staining was performed with Keratocan, CXCL1 and T-cells (anti CD3, rabbit IgG monoclonal SP7) because CXCL1 has been shown to mainly present in T-cells (unpublished data).

For all double staining experiments the sequential alkaline phosphatase (AP) double staining method was used as previously described\(^{12}\). First, Keratocan was visualized with an anti-rabbit IgG/AP labelled polymer (ImmunoLogic) and Vector Blue (Vector Labs, Burlingame, CA, USA). Subsequently, the tissue sections were subjected to an intermediate 10 min, 98\(^\circ\)C heat-induced epitope retrieval step to remove all immunoreagents from the first staining sequence but leaving the Vector Blue reaction product unchanged. Next the slides were incubated with a second primary antibody, followed by an anti-mouse IgG AP-labeled polymer, and visualized with Vector Red (Vector Labs).

Positive controls consisted of samples of human cornea (Keratocan) and human tonsil (cell specific antibodies). Negative controls consisted of experimental tissues stained following the same protocol without the addition of antibodies.

**Western Blots in humans**

Western Blotting was performed to validate the Keratocan expression in human plaque material and corneal tissue (as positive control) found with immunohistochemistry. Cell lysates from atherosclerotic plaque tissue (derived from carotid endarterectomy)
were prepared by collecting cryostat sections (total of 200 µg) in Eppendorf tubes and subsequently adding 400 µl lysis buffer (10 mM Tris HCl, pH 8, 50 mM NaCl, 5 mM EDTA, 1% NP-40, 10% glycerol, 2 mM Na₃VO₄, 5 mM NaF and protease inhibitors). Cell lysates from cornea were prepared by first mechanically crushing the cornea and subsequently adding 400 µl lysis buffer overnight. Fresh cornea tissue was obtained from the Cornea Bank Amsterdam in PBS. Equal amounts of protein were loaded on a NuPAGE® 4-12% Bis-Tris gel (Invitrogen GmbH, Karlsruhe, Germany), separated by electrophoresis and subsequently blotted on a nitrocellulose membrane (Invitrogen GmbH, Karlsruhe, Germany). After blotting, the membranes were blocked in Odyssey® blocking buffer (LI-COR, Nebraska, USA) for 1 h at room temperature. Primary antibody against polyclonal rabbit anti-Keratocan 1:1000 (Rabbit IgG, Sc6694, Santa Cruz Biotechnology, Santa Cruz, California, USA) and monoclonal mouse anti ß-Actin 1:1000 (Abcam, Cambridge, UK) were incubated. Labelled secondary antibody Odyssey® donkey anti mouse IRDye® 800CW and Odyssey® donkey anti rabbit IRDye®, both 1:10.000, (LI-COR, Lincoln, Nebraska, USA) were added. The images were analyzed using the Odyssey Application Software, version 3.0 (LI-COR, Lincoln, Nebraska, USA).

Keratocan expression in an atherosclerotic mouse model

The experiments in animals were approved by the animal review board at Leiden University. In order to study the expression profile of KERA in the process of atherosclerosis immunohistochemistry experiments were performed on carotid arteries with collar-induced atherosclerotic lesions in ApoE knockout mice. Male ApoE knockout mice were fed a Western type diet containing 0.25% cholesterol and 15% cacao butter (Special Diet Services, Sussex, UK) two weeks prior to collar placement surgery and throughout the experiment. Subsets of mice (n=3-4) were anaesthetized at t=0 or at 2, 4, 6, 8 and 10 weeks after collar placement and in situ perfusion-fixation was performed, after which the carotid artery lesions were analyzed. Morphometric analysis (Leica Qwin image analysis software) was performed on hematoxylin-eosin stained 5 µm sections of the carotid arteries at the site of maximal stenosis. KERA expression was visualized by immunohistochemistry with HRP/Nova Red (see supplementary information methods). KERA positive areas were quantified by Leica Qwin image analysis software.

RESULTS

Identification of a non-synonymous variant in KERA

Linkage analysis resulted in the identification of a single 4.44 Mb linkage interval on chromosome 12q21.33-q22 between rs1688545 and rs1493848 markers with a parametric
LOD score of 3.31; an interval harbouring 25 genes (see supplementary information Fig. 1). Sequencing the genomic region within this linkage interval resulted in one novel coding non-synonymous variant in KERA (NM_007035.3: c.920C>G; p.Ser307Cys). This mutation was predicted to be benign according to polyphen, sift and alamut\(^1\)\(^3\),\(^1\)\(^4\). The variant was absent in 9000 healthy controls (1000 Sanquin and 8000 Cambridge Bioresource) and in 1400 early onset CVD patients (1000 AMC-PAS cohort and 400 Boston cohort). Upon sequencing of KERA we did not identify other rare variants in KERA were found in 300 early onset CVD cases.

**KERA, Cornea Plana 2 and CVD**

Ophtalmological evaluation in two carriers of the novel KERA mutation (IV-1en IV-2) showed no signs of CP.

In the 9 CP individuals in the Netherlands, one male underwent CABG at the age of 66 years. None of the first degree relatives of the CP patients had a history of early onset CVD.

**Figure 2.** Keratocan is expressed in atherosclerotic but not normal human coronary arteries

Macrophages are stained red and Keratocan is stained blue. 2a shows a normal coronary artery; macrophages and Keratocan are absent. 2b shows a fatty streak; macrophages entered into the vessel wall and Keratocan is present. 2c shows an atherosclerotic plaque; Keratocan is strongly expressed in the lipid core.
Keratocan expression is associated with atherosclerotic burden in humans
Keratocan was not expressed in mammary arteries and coronary and carotid arteries without atherosclerosis (histomorphic Grade 0). In contrast, in all specimens containing atherosclerotic material, pronounced expression was from early lesions (histomorphic Grade 1) onwards. Keratocan expression in atherosclerotic plaques varied from 10-50% (table 2). Keratocan was most heavily expressed in the extracellular matrix of the lipid core.

In both early and progressed atherosclerotic lesions Keratocan was expressed in endothelial and smooth muscle cells, but most prominent in macrophages. Co-localisation studies showed that T-cells were positive for both KERA and CXCL-1 (see supplementary information: figure 2). KERA Western blots proved the presence of KERA in the cornea and plaque in plaque tissues (see supplementary appendix: figure 3).

Keratocan expression is associated with atherosclerotic burden in mice
Also in mice Keratocan expression was related to atherosclerosis progression (see supplementary information: figure 4) Perivascular collar placement in the Western type diet fed ApoE Knock Out (KO) mice induced atherosclerosis. The extent of atherosclerotic lesion formation, being quantified by intima thickness was significantly correlated with the expression of Keratocan measured by surface area. ($r^2 = 0.7$, $p < 0.001$) (Figure 3a and b). Keratocan was mainly localized in necrotic areas, endothelial cells and smooth muscle cells. Co-localisation with macrophages was less compared to the human sections.

Figure 3. Expression of Keratocan in ApoE KO mice after induction of atherosclerosis

Keratocan expression showed an increase in time after collar placement and significant correlation with plaque size.
DISCUSSION

We identified a novel non-synonymous rare variant in KERA in a large family with a Mendelian form of early onset coronary and carotid atherosclerotic disease by linkage analysis and next generation sequencing. The variant was not present in 9000 healthy controls and 1400 patients with premature CVD. KERA encodes for Keratocan, a keratan sulfate proteoglycan, expressed in the cornea, trachea and at lower levels in the intestine, skeletal muscle, ovary and lung\textsuperscript{15}. The protein was absent in healthy artery segments, but heavily abundant within the lipid core of atherosclerotic lesions, which emphasizes that Keratocan might be a novel actor in the pathological process underlying atherosclerosis. This notion is further strengthened by the fact that in ApoE knockout mice, induction of atherosclerosis in the carotid arteries by cuff placements significantly correlated with Keratocan expression in the plaques.

Thus far, loss-of-function mutations in KERA have been described as a cause for cornea plana 2 (CP2) (OMIM 121400; 217300), a rare autosomal recessive disorder, characterized by excessively flat and thin corneas and peripheral or central scleralization. Worldwide only 84 cases have been described\textsuperscript{16, 17}. An increased risk for CVD in these individuals has not been reported, but this might have resulted from the fact that previous studies have focussed on the ophthalmological consequences of KERA mutations. In our study, however, we did not observe early onset CVD in the nine identified CP patients with cornea plana in the Netherlands, nor in their first degree relatives. The absence of corneal manifestations in 2 affected family members (IV-I and IV-2) carrying the novel KERA mutation emphasizes the clinical diversity between heterozygous carriers of our novel variant and carriers of other heterozygous variants resulting in CP.

Keratocan has been shown to play a role in neutrophil migration after corneal injury\textsuperscript{18}. In mice, neutrophil migration is orchestrated by a chemical gradient in the vessel wall of a wide range of cytokines including CXCL1\textsuperscript{19}. Keratocan is one of the regulators of the CXCL1 gradient\textsuperscript{20}. The receptor for CXCL1, CXCR2, is present on neutrophils and on monocyte/macrophages, which are directly involved in all sequelae of atherosclerosis\textsuperscript{17}. We hypothesize that a gain of function mutation in our kindred results in an augmented binding of Keratocan to CXCL1 and an increased neutrophil migration into the vessel wall. In mice, both arterial CXCL1 and leukocyte-specific CXCR2 expression are central to macrophage accumulation in established fatty streak lesions\textsuperscript{21} and in mice lacking CXCL1 atherosclerosis is significantly reduced\textsuperscript{22}. Further studies are warranted to confirm our hypothesis.

A number of considerations have to be taken into account while interpreting the data. The results from this study are highly suggestive of a putative role for Keratocan in the process of atherosclerosis, but a direct causative role has not been established thus far.

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This direct causal effect can be studied in mice models, where on a proatherogenic background (apoE KO) overexpression of Keratocan should conceptually result in a more proatherogenic phenotype, mimicking the effect of our putative gain of function mutation.

Another item that has to be addressed is the remarkable difference in clinical phenotypes in CP patients, who are either compound heterozygous/homozygous for KERA mutations, and the heterozygous carriers who suffered from CVD in our study warrants further investigation.

In addition, we did not identify any additional carriers of mutations in KERA in 300 individuals with premature CVD, which precluded us from doing additional studies in other families. However, if through the discovery of this very rare variant, we have identified an additional pathway which might increase our comprehension of atherosclerosis and ultimately result in novel targets for therapy.

The application of a combined linkage analysis and next generation targeted sequencing approach is relatively novel and has not been used in families with CVD. In previous family studies there was a biased selection of genes to be followed up by sequencing. Since it was technically not possible to sequence whole intervals candidates were chosen based on the literature. This resulted in the identification of genes such as MEF2A and LRP6. With the application of next generation sequencing, we have applied an unbiased approach, to identify novel variation in this family.

The sheer size of our family facilitated gene discovery. However we realize that nowadays it is challenging to identify such large families with a clear Mendelian inheritance pattern of early onset CVD.

In conclusion, the current study for the first time shows a strong association between Keratocan and atherosclerosis, and further studies will have to be carried out to address the causality.

ACKNOWLEDGEMENTS

This project was funded by Bloodomics consortium, European Union 6th Framework Programme (LSHM-CT-2004-503485) and Ipse Movet. JK is holder of the Lifetime Achievement Award of the Dutch Heart Foundation (2010T082). WHO is supported by program grants from the British Heart Foundation and the National Institute for Health Research, England. GKH is holder of a Veni grant (91612122) from the Netherlands Organisation for Scientific Research (NWO). We would like to acknowledge Christy Holtkamp for the genetic fieldwork and Jitske Kuipers and Caroline Aalbers for technical and laboratory assistance.
REFERENCES


### Supplementary Table 1. Clinical Characteristics Family

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**Abbreviations**
- CVD: Cardiovascular Disease
- yrs: Years
- BMI: Body Mass Index
- TC: Total Cholesterol
- LDL-c: Low-Density-Lipoprotein-Cholesterol
- HDL-c: High-Density-Lipoprotein-Cholesterol
- TG: Triglycerides
- Simva: Simvastatin
- Prava: Pravastatin
- Atorva: Atorvastatin
- Rosuva: Rosuvastatin
**Supplementary Table 2. Quantification of co-localisation of KERA with CD68, CD34 and SMA**

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<th>Grade of lesion</th>
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* Grade of lesion: 0: no lesion or intimal thickening, corresponding with macroscopically normal arterial wall (AHA classification type 1 n=5); 1: fatty streaks (AHA type II, n=5). 2: lipid rich plaques (AHA type IV, Va), and fibrous plaques (AHA type Vb, c) in total n=10, 3: thrombosed plaques (AHA type VI, n=3); aKERA grade: 0= Not present, 1= <10% of the plaque, 2= 10-50% of the plaque, 3=>50% of the plaque; CD34: Microvessels and subpopulation of endothelium were considered; dIf double stained CD68+ cells are present, it includes only a subpopulation of the macrophages and faint staining. In 75% of the Grade 1-3 lesions, where macrophages were present, Keratocan clearly co-localized with a subpopulation of macrophages. For endothelial cells and smooth muscle cells these number were lower and respectively; 44% and 31%.
A significant LOD score of 3.31 was obtained on chromosome 12 after parametric linkage analysis by Allegro software using genotypes from 12 available family members.

Supplementary Figure 2. Triple staining for Keratocan, CXCL-1 and CD3+.

Triple staining for CXCL-1 (red); KERA (blue) and CD3+ cells (brown) demonstrate that these cells are positive for all three components. As described in the literature CXCL-1 is clearly present in the atherosclerotic vessel wall.
**Supplementary Figure 3.** Western blot of Keratocan in human plaques and cornea

![Western blot image](image)

Keratocan was seen as a ~53kDa band after western blotting using Keratocan antibody. Cornea tissue was used as positive control.

**Supplementary Figure 4.** Keratocan is present in atherosclerotic mice

Early and advanced atherosclerotic tissue from murine carotid arteries were stained using Keratocan antibody (brown) and Hematoxylin as a counterstaining (blue). While present mainly near endothelial cells in early lesion, Keratocan is predominantly present in the matrix of the plaque in advanced lesions, suggesting production by endothelial cells and secretion in the matrix.
SUPPLEMENTARY METHODS

CVD Definition
CVD was defined by the presence of at least one of the following: (I) myocardial infarction, proven by at least two of the following: (a) classical symptoms (>15 min), (b) specific electrocardiographic abnormalities, (c) elevated cardiac enzymes (>2× upper limit of normal); (II) percutaneous coronary intervention or other invasive procedures; (III) coronary artery bypass grafting; (IV) angina pectoris, diagnosed as classical symptoms in combination with at least one unequivocal result of one of the following: (a) exercise test, (b) nuclear scintigram, (c) dobutamine stress ultrasound, (d) a more than 70% stenosis on a coronary angiogram or (f) requiring treatment(V) ischemic stroke, demonstrated by CT- or MRI scan; (VI) documented transient ischemic attack.

GENETICS

Targeted sequencing
A Nimblegen custom sequence capture with 395K probes was designed for the target region of 4465094 bases. A total of 80.7% of the target bases were covered by a probe. Of the 240 coding exons in the target region, one exon was not covered by the design and two were partially contained. In total there were 340 exons in the target region, of which 56 were partially covered and 12 were more than 500 bp from a tile boundary. The sequence capture was performed once using array-based capture and once using solution-based capture.

Respectively 2.6 and 1.8 Gb of Illumina GAII sequencing using 54 bp paired-end reads was generated for the two sequence captures, yielding an average total coverage in the target region of 693X. Reads were aligned to the Hg18 reference genome using BWA (http://www.ncbi.nlm.nih.gov/pubmed/20080505), and duplicates were removed using Picard (Web-based application Picard. http://picard.sourceforge.net (Accessed 12/2010.). SNPs were called using the SAMtools ‘pileup’ method’, and a simple base counting method whereby each position with at least three bases not matching the reference was called as a SNP. Small insertions and deletions were called using Dindel. Variants were called for the BAM file for each sequence capture independently, and then results were merged. Any identified non-synonymous rare variant was checked for presence in dbSNP, the available data from the 1000 genomes project2.
Probes and primers used for genotyping and sequencing

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COHORTS

Patient cohorts
AMC-PAS cohort is a prospective study cohort recruited from the Academic Medical Centre Amsterdam and consists of patients with Premature Atherosclerosis (PAS), defined as symptomatic CAD before the age of 51 years, defined as MI, coronary revascularization, or evidence of at least 70% stenosis in a major epicardial artery.
Boston Cohort is a prospective study cohort recruited from the MGH in Boston and consists of patients with Early Onset CVD, defined as symptomatic CAD before the age of 51 years, defined as MI, coronary revascularization, or evidence of at least 70% stenosis in a major epicardial artery.

Blood donor cohorts
Sanquin cohort is a collection of 1000 DNA samples from blood donors recruited at routine Sanquin Blood Bank donation sessions. More than 95% of the controls are from the same region as the cases of the AMC-PAS cohort.
Cambridge BioResource cohort is a collection of 8000 pseudo-anonymised DNA samples off healthy blood donors established by the Cambridge Biomedical Research Centre in collaboration with NHS Blood and Transplant for use in genotype-phenotype association studies.

Immunohistochemistry in mice
Cryostat sections of the aortic root (n=7; 10 mm) were collected and embedded in Tissue Tek (Sakura Finetek, Alphen aan de Rijn, The Netherlands). The slides were washed in PBS to remove Tissue Tek. Subsequently slides were blocked with 5% milk powder. Next, the slides were incubated with anti-KERA 1:1000 (rabbit IgG, Sc6694 Santa Cruz Biotechnology, Santa Cruz, California, USA), followed by anti-rabbit IgG/AP-labeled polymer (ImmunoLogic, Duiven, The Netherlands). HRP activity was demonstrated by red staining with NOVA RED (Vector Laboratories, Burlingame, California, USA). The slides were counterstained with hematoxyline.
REFERENCES