

Altered Fibrin Clot Structure in the Healthy Relatives of Patients With Premature Coronary Artery Disease

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Background—A family history of premature coronary artery disease (CAD) is an independent cardiovascular risk factor. Fibrin clots composed of dense fiber networks are found in young CAD patients and may occur in the relatives of such individuals.

Methods and Results—The ex vivo fibrin structure of 100 healthy male relatives of patients with premature CAD and 100 age-matched control subjects was assessed by measurement of permeability (K_s), fiber mass-length ratio (μ), and turbidity (lag phase and maximum absorbency [max Δ Abs]). Scanning electron microscopy was performed on selected samples. Relatives and controls shared similar levels of conventional cardiovascular risk factors. K_s was lower in relatives than in controls, 12.2 (11.1 to 13.3) versus 15.2 (14.0 to 16.5) $\times 10^{-9}$ cm² ($P < 0.001$), associated with a smaller decrease in μ , 8.5 (7.7 to 9.2) versus 9.7 (8.9 to 10.5) $\times 10^{13}$ Da/cm ($P < 0.05$), respectively. Lag phase was shorter in relatives than in controls, 39 (37 to 41) versus 47 (44 to 50) seconds ($P < 0.001$), and max Δ Abs was higher in relatives, 0.78 (0.74 to 0.82) versus 0.71 (0.67 to 0.74) in controls ($P = 0.02$), which indicates the presence of thicker fibers in relatives. After adjustment for fibrinogen levels, lag phase and K_s remained significantly different between relatives and control subjects. Scanning electron microscopy images confirmed increased fiber diameter in relatives, possibly of reduced density. Factor XIII Val34Leu and fibrinogen A α Thr312Ala and B β -455 G/A showed no association with clot structure.

Conclusions—The male relatives of patients with premature CAD form fibrin clots that begin polymerization more quickly, have thicker fibers, and are less permeable than those of control subjects. (*Circulation*. 2002;106:1938-1942.)

Key Words: fibrin structure ■ fibrinogen ■ coronary disease ■ thrombosis ■ fibrinolysis

There is consensus that fibrinogen, tissue plasminogen activator antigen (tPA), plasminogen activator inhibitor 1 (PAI-1), and fibrin D-dimer are risk factors for the progression and development of coronary artery disease (CAD).¹ The final product of coagulation is the formation of a fibrin clot, the structure and function of which is modulated by genetic and environmental factors that regulate the hemostatic system. Relatives of subjects with CAD are at increased risk of vascular disease,² and alterations in fibrin structure/function may contribute to vascular risk in such individuals.

We performed a case-control study to investigate the structure of ex vivo fibrin clots from healthy male first-degree relatives of patients with severe CAD. The relationship between hemostatic factors, their genetic polymorphisms, and the biophysical properties of these fibrin clots was assessed.

Methods

Subjects

One hundred male first-degree relatives aged 65 years or less at the time of recruitment and free from a personal history of CAD were enrolled in the study. These subjects were the healthy relatives of

patients (probands) with angiographically confirmed 2- or 3-vessel CAD based on World Health Organization criteria of $>50\%$ stenosis in a major epicardial vessel. Probands were identified via the surgical revascularization waiting list at the Yorkshire Heart Center, Leeds, and their clinical characteristics were as follows: median age 59 years (interquartile range 52 to 66 years); 52% had suffered a myocardial infarction; 30% were hypertensive; 12% had type 2 diabetes; and 94% were taking aspirin on a regular basis. Each of the 100 probands identified had a relative suitable for enrollment in the study. Additionally, 100 healthy male subjects aged 65 years or less without a personal or family history of CAD or diabetes mellitus were recruited from the Leeds Health Authority Family Health Service registry. Controls were matched to the first-degree relatives.

Ethical Consideration

All subjects were white and North European and gave informed consent according to a protocol approved by the Leeds Teaching Hospitals (NHS) Trust Research Ethics Committee.

Blood Sample and Data Collection

After a 10-hour overnight fast, 50 mL of blood was taken from an antecubital vein without stasis by use of a 19-gauge needle with the subject in a supine position. Blood was collected in lithium fluoride for plasma glucose estimation, lithium heparin for lipid fraction

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analysis, EDTA for DNA extraction, and in a 10-mL tube containing 1 mL of 0.9% citrate (pH 8.8) at room temperature for assay of fibrinogen, fibrin structure, and factor XIII (FXIII) and at 4°C for assay of tPA, PAI-1, and fibrin D-dimer. Citrate samples were centrifuged at 2560g for 20 minutes, and aliquots of 0.5 mL of plasma supernatant were snap-frozen in liquid nitrogen for storage at -40°C until assay. Blood pressure was measured to the nearest 2 mm Hg and calculated as a mean of 3 consecutive readings. Relatives and control subjects with a personal history of hypertension were excluded from subsequent blood pressure analysis.

Hemostatic Factor Measurement

Fibrinogen was measured as described previously.³ PAI-1, tPA, and fibrin D-dimer were measured with commercially available ELISA kits from Biopool International. FXIII A₂B₂ and B-subunits were determined by sandwich ELISA as described previously.⁴ A glucose oxidase method was used for measurement of plasma glucose, and a Hitachi 747 autoanalyzer was used for estimation of triglyceride and total cholesterol. HDL cholesterol was measured by a Hitachi 717 autoanalyzer after precipitation of LDL, chylomicrons, and VLDL with phosphotungstic acid and magnesium chloride. LDL cholesterol was calculated by the Friedewald equation.

Genotyping

Genotypes at the fibrinogen B β -455 G/A polymorphism (classified as G/G, G/A, and A/A), fibrinogen A α Thr312Ala (TT, TA, and AA), and FXIII Val34Leu polymorphism (V/V, V/L, and L/L) were analyzed as described previously.^{3,5,6}

Fibrin Permeation Analysis

Plasma samples were incubated with 1 U/mL human thrombin (Sigma) and 20 mmol/L calcium in open tubes for 2 hours at room temperature in a wet chamber. The tubes containing the clots were connected via plastic tubing to a reservoir containing 0.05 mol/L Tris-HCl, 0.15 mol/L NaCl, pH 7.5, with a pressure drop of 4 cm. After washing, flow rates of buffer through the fibrin clots were measured by timing the permeation of 6 consecutive drops through each tube and recording the weight of each drop for exact volume. The Darcy constant, K_s , which represents the surface of the clot that allows flow through a fibrin network, was calculated as described previously.⁷ Fiber mass-length ratio (μ) was calculated from an equation involving K_s and the fibrinogen molar concentration.⁸

Turbidity Measurements

Plasma, diluted 2/3 with 0.05 mol/L Tris-HCl, 0.15 mol/L NaCl, pH 7.5, was incubated with 1 U/mL human thrombin (Sigma) and 16 mmol/L calcium in microtiter plates. Immediately on addition of thrombin/calcium, absorbency was read every 7 seconds at 350 nm for 15 minutes with a Dynex MRX2 plate reader. Lag phase, which is the time required for fibrin protofibrils to grow to sufficient length to allow lateral aggregation to occur and which is sensitive to a variety of factors including fibrinogen concentration and rate of fibrinopeptide A cleavage,⁹ was recorded as the time taken for absorbency to change by 0.01 from baseline. Maximum absorbency (max Δ Abs), which has been shown to reflect average fibrin fiber size and therefore the number of protofibrils per fiber,⁹ was recorded as absorbency at 15 minutes minus the baseline. Turbidity curves reached a plateau within 15 minutes of analysis, and 2 replicate measurements were performed for each sample.

Scanning Electron Microscopy

Samples were selected from 8 subjects with fibrinogen concentrations of high and low values relative to the study group means, ie, 2 relatives and 2 controls each with a concentration of 3.3 g/L and 2 relatives and 2 control subjects with a concentration of 2.6 g/L. Clots were fixed by permeating them with a 2% (vol/vol) glutaraldehyde solution overnight, recovered from the tubes and further processed by dehydration.^{7,9} Samples were photographed digitally in 6 different areas with a Camscan IV scanning electron microscope (SEM) and secondary electron imaging for topographic contrast. To assess

TABLE 1. Clinical and Biochemical Data

	Relatives (n=100)	Controls (n=100)
Age, y	34 (20–48)	38 (23–52)
Current smokers, %	27	26
Cigarettes per day	16 (13–19)	17 (12–22)
Alcohol, U/wk	21 (18–25)	20 (16–24)
Body mass index, kg/m ²	25.7 (25.0–26.4)	25.7 (25.0–26.3)
Waist-hip ratio	0.89 (0.89–0.91)	0.90 (0.89–0.91)
Systolic BP, mm Hg	137 (134–140)	134 (131–137)
Diastolic BP, mm Hg	81 (79–84)	81 (78–83)
Total cholesterol, mmol/L	5.2 (5.0–5.4)	5.1 (4.9–5.3)
HDL cholesterol, mmol/L	1.3 (1.3–1.4)	1.3 (1.2–1.4)
LDL cholesterol, mmol/L	3.1 (2.9–3.3)	3.1 (2.9–3.3)
Triglyceride, mmol/L	1.4 (1.2–1.6)	1.3 (1.2–1.5)
Fasting glucose, mmol/L	5.0 (4.9–5.1)	5.0 (4.9–5.1)

Values for age are median (25th and 75th percentiles); body mass index, waist-hip ratio, systolic BP, diastolic BP, and HDL, LDL, and total cholesterol are mean (95% CI); and for the remainder, values are geometric mean (95% CI). BP indicates blood pressure.

fiber thickness, a series of 50 x/y coordinates were generated randomly and applied to each SEM image. An independent observer measured fiber diameter at or nearest to each of the 50 coordinates. Only those fibers with clearly defined margins were measured. Similarly, 10 sets of randomly generated coordinates were used to assess the number of fibers per 4- μ m² SEM image area. All measurements were performed with Image J version 1.25s software (W. Rasband, National Institutes of Health).

Statistical Analysis

Values for age are presented as medians with 25th and 75th percentiles, and differences were assessed by the Kruskal-Wallis test. Values for fibrinogen, tPA, PAI-1, D-dimer, plasma fasting glucose, and triglycerides were log transformed, and data are presented as geometric means with antilog 95% CIs. An independent Student's *t* test was used to assess differences in continuous data, and a χ^2 test was used for differences in category frequencies. One-way ANOVA was used to identify any relationship between genotype and coagulation factor level and measures of clot structure/function. Spearman correlation coefficients were calculated to identify significant associations of K_s , lag phase, and max Δ Abs with hemostatic factors and other continuous variables. Multiple linear regression analysis was performed for K_s , lag phase, and max Δ Abs, and the data presented represent the best-fit model as determined by the R^2 value. Analyses were performed with SPSS version 9.0.

Results

Clinical and biochemical data for relatives and controls are shown in Table 1. Age, lifestyle habits, and conventional cardiovascular risk factors were not different between relatives and control subjects. Three relatives and 2 controls were taking either a β -blocker or an ACE inhibitor for hypertension. Exclusion of these subjects did not alter the results of the study, and they were therefore included in all analyses except SEM.

Hemostatic factor levels and data concerning ex vivo fibrin clot structure are shown in Table 2. Compared with control subjects, relatives produced fibrin clots that began formation more quickly (shorter lag phase), showed a greater change in absorbency (max Δ Abs), and contained pores of a smaller

TABLE 2. Hemostatic Factor Levels and Fibrin Clot Structure Data

	Relatives (n=100)	Controls (n=100)
Fibrinogen, g/L	3.1 (3.0–3.2)*	2.8 (2.7–2.9)
Fibrin D-dimer, ng/mL	52 (47–57)	52 (46–59)
tPA antigen, ng/mL	7.8 (7.1–8.5)†	5.4 (4.8–6.1)
PAI-1 antigen, ng/mL	12.0 (9.9–14.6)	11.5 (9.6–13.7)
FXIII A ₂ B ₂ , U/mL	1.23 (1.17–1.30)	1.12 (1.07–1.19)
K _s ×10 ⁻⁹ , cm ²	12.2 (11.1–13.3)†	15.2 (14.0–16.5)
μ (Mass: length ratio)×10 ¹³ , Da/cm	8.5 (7.7–9.2)‡	9.7 (8.9–10.5)
Lag phase, s	39 (37–41)†	47 (44–50)
Max ΔAbs (350 nm)	0.78 (0.74–0.82)‡	0.71 (0.68–0.74)

Values for FXIII A₂B₂, K_s (Darcy constant), μ (mass: length ratio), lag phase, and max ΔAbs are mean (95% CI); the remainder are geometric mean (95% CI). FXIII A₂B₂ concentration is in U/mL, where 1 U/mL is defined as the concentration in a pool of normal plasma.

*P≤0.01; †P<0.001; ‡P<0.05.

size (lower K_s). Fiber mass-length ratio (μ) was also lower in relatives. Fibrinogen and tPA antigen levels were increased in relatives compared with controls. A nonsignificant trend toward elevated levels of FXIII A₂B₂ was observed in the relatives.

Fibrinogen demonstrated a strong and consistent relationship with all measures of clot structure in both groups, except lag phase in controls (Table 3). D-dimer was positively correlated with max ΔAbs in relatives and controls and negatively correlated with K_s in relatives. tPA displayed a negative association with lag phase in control subjects, but neither PAI-1 nor FXIII A₂B₂ was associated with measures of fibrin clot structure. In relatives and controls, K_s was negatively correlated with max ΔAbs and positively correlated with lag phase (data not shown).

The final linear regression analysis model for K_s is shown in Table 4. Only fibrinogen levels and being a relative of a CAD patient were independent predictors of K_s. Mean values for K_s adjusted for age, fibrinogen, max ΔAbs, and lag phase, were 12.9×10⁻⁹ (11.7 to 14.0) cm² in relatives versus 14.6×10⁻⁹ (13.5 to 15.7) cm² in controls (P<0.05). Fibrino-

TABLE 4. Multiple Linear Regression Model Incorporating Relatives and Control Subjects With K_s as the Dependent Variable

Parameter	Coefficient (95% CI)	Contribution to Variance, %	Significance (P)
Final model		21.9	<0.001
Log fibrinogen	-15.3 (-27.8 to -2.7)	3.8	<0.05
Relative (vs control)	-1.7 (-3.4 to -0.7)	2.8	<0.05
Max ΔAbs	-4.5 (-11.0 to 2.1)	1.2	NS
Age	-0.5 (-0.1 to 0.0)	1.0	NS
Lag phase	0.0 (-0.1 to 0.1)	0.5	NS

NS indicates not significant.

gen concentration and relative/control subject status were significant predictors of fiber mass-length ratio. Similar regression analysis was performed for lag phase, which resulted in adjusted mean lag phase times of 40 (38 to 43) seconds in relatives and 46 (43 to 48) seconds in control subjects (P<0.01). After adjustment for age, fibrinogen, K_s, and lag phase, being a relative was no longer associated with increased max ΔAbs.

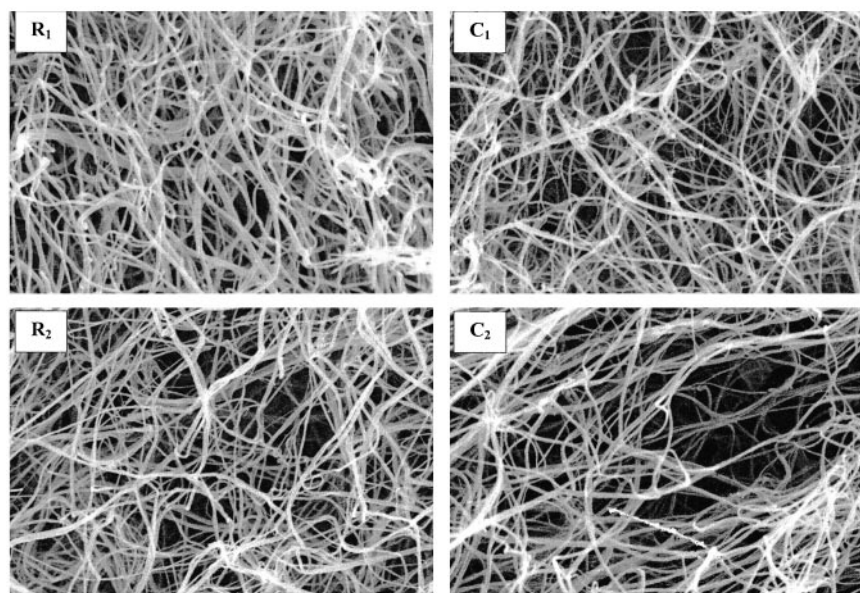
Digital images from a representative selection of SEMs obtained are shown in the Figure. Panels R₁, R₂, C₁, and C₂ represent 4 different fibrin clots, with the clots in the upper panels (R₁ and C₁) produced from plasma samples containing 3.3 g/L fibrinogen compared with 2.6 g/L of fibrinogen in the plasma used to produce the fibrin clots in the corresponding lower panels (R₂ and C₂). Overall fiber diameter was greater in relatives than in controls, with a mean thickness of 202 (187 to 218) versus 166 (157 to 177) nm (P<0.001) for high fibrinogen samples and 174 (161 to 187) versus 154 (144 to 164) nm (P=0.02) for low fibrinogen samples. High fibrinogen concentration was associated with increased fiber diameter in relatives (P=0.005) but did not reach statistical significance in controls (P=0.07). There were no observed differences in fiber number between relatives and controls or between the clots of high and low fibrinogen concentration.

Genotype frequencies for FXIII Val34Leu and fibrinogen Aα Thr312Ala were similar in relatives and controls. No clear or consistent relationship between genotype and any of the measures of fibrin clot structure was found.

TABLE 3. Bivariate Correlation Coefficients for K_s, Max ΔAbs, and Lag Phase

	Controls			Relatives		
	K _s	ΔAbs	Lag	K _s	ΔAbs	Lag
Age	-0.22	0.12	-0.18	-0.19	0.34	-0.29
Waist-hip ratio	-0.19	0.17	-0.15	-0.24	0.40	-0.28
Systolic blood pressure	-0.25	0.01	-0.11	-0.21	0.04	0.01
HDL cholesterol	0.01	-0.05	0.06	0.17	-0.23	0.30
Fibrinogen	-0.32	0.62	0.01	-0.41	0.77	-0.45
tPA antigen	-0.12	0.15	-0.23	-0.07	0.10	-0.01
PAI-1 antigen	-0.13	0.09	0.07	-0.16	0.02	-0.16
Fibrin D-dimer	-0.19	0.27	0.09	-0.26	0.32	-0.13
FXIII A ₂ B ₂	-0.14	0.20	0.05	0.14	0.01	0.02

P<0.05 where |r|≥0.22.



Representative SEMs from relatives (R_1 , R_2) and controls (C_1 , C_2). R_1 and C_1 are from plasma samples containing same high fibrinogen concentration, and R_2 and C_2 are from samples with low fibrinogen level. R_1 and R_2 demonstrate reduced porosity and increased fiber diameter compared with C_1 and C_2 , which results in appearance of more densely packed fibrin fiber network in R images.

Discussion

We have shown that the plasma from healthy male first-degree relatives of patients with premature CAD form ex vivo fibrin clots with abnormal structure. These clots were less porous, demonstrated a lower fibrin fiber mass-length ratio, contained thicker fibers, and began polymerization more quickly than those of a well-characterized and highly comparable group of control subjects. SEM images provided visual and additional quantitative information concerning the differences in fiber thickness between the 2 groups, although it is important to note that the SEMs were obtained from only a limited number of samples.

The few clinical studies that have examined fibrin structure/function have produced inconsistent findings. Greulich et al¹⁰ examined clot elastic modulus and fiber mass-length ratio in 14 patients with CAD and 10 healthy controls. Despite aspirin therapy, patients with severe CAD had more rigid clot structures and an elevated fiber mass-length ratio.¹⁰ In a further report, the fibrin gel structure of patients with type 1 diabetes was found to be less porous than that of nondiabetic controls, but this was not related to fibrinogen levels, and there was no difference in fiber mass-length ratio.¹¹ Fatah et al^{12,13} identified both reduced permeability and fiber mass-length ratio in the fibrin gels from young patients after myocardial infarction. Independent associations between K_s and extent and severity of coronary artery stenosis¹² and between K_s and fibrinolytic activity¹³ were also reported. There are no previous studies that investigated fibrin clot structure in the healthy relatives of CAD patients, nor are there any reports that have included permeation, turbidity measurements, and SEM images to visualize clot structure differences.

We found decreased fibrin clot permeability in first-degree relatives of patients with CAD, a finding consistent with those of Fatah et al^{12,13} in patients with myocardial infarction.

We also found a decrease in fiber mass-length ratio, but turbidity measurements and analysis by SEM showed an increase in fiber thickness. Fiber mass-length ratio is calculated from the permeation constant, fibrin concentration, and fiber density, the latter of which, however, could not be measured in the present study and to which an assumed value had to be given to calculate μ .⁸ Our finding of reduced mass-length ratio with increased diameter suggests that the fibrin fibers could be thicker but less dense in first-degree relatives. However, because μ is a calculated value from a number of measures, and methodological inconsistencies and variation may be reflected in this parameter, any deduction regarding fiber density has to be made cautiously.

Although the mechanism by which elevated fibrinogen translates to a higher incidence of coronary disease is not known, fibrinogen concentration has a profound effect on fibrin clot structure. Fibrin polymerization and protofibril assembly begin as thrombin cleaves fibrinopeptide A from fibrinogen, with a time course in the magnitude of seconds. The rate of fibrinopeptide A cleavage increases with fibrinogen concentration,¹⁴ and a faster activation rate is associated with a shorter lag phase and a more dense and tight fibrin network.¹⁵ In addition, elevated fibrinogen levels lead to the formation of thicker fibers, as estimated from turbidity studies.^{16,17} We have shown that fibrin clots from relatives of patients with premature CAD are formed after a shorter lag phase, have greater max ΔAbs , and have lower K_s and μ values than with controls and that these components of clot formation and structure are strongly correlated with plasma fibrinogen levels. However, in a linear regression model, only the difference in max ΔAbs could be fully accounted for by the difference in fibrinogen concentration between the 2 groups. The association between family history of premature CAD and low K_s , low μ , and short lag phase remained independent of adjustment for fibrinogen and other signifi-

cant correlates. These data suggest that other as yet unidentified genetic or environmental factors may affect fibrin clot structure in families in whom severe CAD occurs prematurely.

FXIII plays an important role in fibrin cross-linking. A Val34Leu polymorphism in the FXIII A-subunit has been associated with thrombotic disease and results in an amino acid substitution in close proximity to the thrombin activation site. In addition, a polymorphism in the A α chain of the fibrinogen gene, Thr312Ala, lies between the residues involved in α - α and α -antiplasmin cross-linking. Data from our unit suggest a significant interaction between fibrin clot porosity and turbidity measurements and A α Thr312Ala (unpublished data) and Val34Leu genotype.⁸ In the present study, we did not find such a relationship. It is possible that the profound effect of fibrinogen concentration on clot structure and the wide range of fibrinogen levels would mask associations between a single polymorphism and altered clot structure. Nevertheless, given the independent relationship between relatives of CAD patients and reduced K_s values, genetic factors may well be important in the determination of fibrin clot structure.

A relationship between fibrin clot structure and fibrinolysis has been reported in several studies. The majority of data suggests a link between dense, fine fibrin networks and hypofibrinolysis, either in terms of a strong negative association between PAI-1 activity and K_s ¹³ or a directly measured reduction in lysis rate.^{18–20} A recent report by Collet et al²¹ using a dynamic confocal microscopy technique confirmed reduced lysis front velocity in tight, thin fiber meshworks compared with clots of thicker, looser fibers. However, the lysis rates of individual fibers within the clots were also measured, with the finding that thicker fibers were lysed more slowly than thin ones. Such data imply that the changes observed in the present study might increase resistance to fibrinolysis and enhance the thrombogenic potential of the altered fibrin clot structures observed in healthy relatives of patients with CAD. Platelets and other cells interact with fibrin and play an important role in the formation of the blood clot. Cellular interaction has important consequences for the speed at which fibrinolysis proceeds,²² and the implications of the present findings for the stability of the clot will depend in part on this.

Formation of a fibrin clot is the end point of coagulation. We have shown that the healthy male relatives of patients with severe premature CAD form less porous fibrin clots that contain thicker fiber networks than those of healthy subjects free from a family history of coronary disease. These findings represent a plausible mechanism by which a family history of premature CAD exerts its deleterious effects on an individual's risk factor profile. Low-dose aspirin has been shown to increase fibrin gel porosity in healthy subjects,²³ and such interventions may be indicated in the relatives of patients with premature CAD, even in the absence of other cardiovascular risk factors. At the present time, the implications of these data remain speculative, and prospective studies are required to establish the role of altered fibrin clot structure in the development of clinically apparent CAD.

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