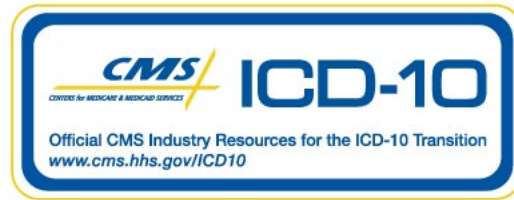


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Endovascular Presence of Viable *Chlamydia pneumoniae* Is a Common Phenomenon in Coronary Artery Disease

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Objectives. We sought to examine coronary arteries for the presence of viable bacteria of the fastidious species *Chlamydia pneumoniae*.

Background. The respiratory pathogen *C. pneumoniae* has been implicated in the pathogenesis of coronary artery disease (CAD). Previous studies have demonstrated an antichlamydial seroreponse to be a cardiovascular risk factor and coronary atheromata to contain chlamydial components in varying proportions. Endovascular demonstration of replicating bacteria is required to provide evidence for an infectious component in CAD and a rationale to discuss antimicrobial therapy.

Methods. Myocardial revascularization was performed in 70 patients. Atherosclerotic lesions from 53 coronary endarterectomy and 17 restenotic bypass samples were cultured and subjected to nested polymerase chain reaction (PCR) for *C. pneumoniae*. Antichlamydial immunoglobulin G (IgG), IgA and IgM was examined by microimmunofluorescence.

Results. Viable *C. pneumoniae* was recovered from 11 (16%) of 70 atheromata, and chlamydial deoxyribonucleic acid (DNA) was

detected in 21 (30%) of 70 atheromata; 17 nonatherosclerotic control samples were PCR-negative ($p < 0.01$). Fifteen (28%) of 53 endarterectomy and 6 (35%) of 17 bypass samples were PCR-positive. DNA sequencing of six different PCR products did not reveal differences between coronary isolates and respiratory reference strains, suggesting that common respiratory strains gain access to the systemic circulation. Serologic results did not correlate with direct detection results and did not identify individual endovascular infection.

Conclusions. A significant proportion of atherosclerotic coronary arteries harbor viable *C. pneumoniae*. This finding supports the hypothesis of a chlamydial contribution to atherogenesis. Whether chlamydiae initiate atherosclerotic injury, facilitate its progression or colonize atheromata is unknown. However, the endovascular presence of viable bacteria justifies a controlled clinical investigation of antimicrobial treatment benefit in the therapy and prevention of CAD.

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Chlamydia pneumoniae has been established as an important respiratory pathogen (1-3). The obligate intracellular bacterium is characterized by its extraordinarily high prevalence: seroepidemiologic results indicate that virtually everyone becomes infected at least once during their lifetime (2). As chlamydiae are notorious for causing persistent disease with severe tissue destruction, concern about sequelae from recurrent chlamydial infection is justified. In this respect, Saikku et al. (4) related chronic coronary artery disease (CAD) with previous or persistent *C. pneumoniae* infection. Based on antichlamydial immunoglobulin G (IgG) elevation and the presence of immune complexes with chlamydial lipopolysaccharides in patients with CAD, *C. pneumoniae* infection was

suggested as an independent cardiovascular risk factor (4,5). After initial concerns, these surprising results were reproduced wherever the attempt was made in a microimmunofluorescence test system (6-8), as well as by immunoblot procedures (9). However, the indirect statistical associations could not be taken as evidence of causality.

Endovascular infection might provide an explanation for unclear phenomena of atherogenesis, like mesenchymal cell proliferation and the distinct inflammatory component (10). A contribution of *C. pneumoniae* in this respect requires its presence in diseased arteries, and several investigators have reported an occurrence of pathogen-specific nonviable structures in atheromatous plaques, although detection rates varied widely with the techniques employed (11-13). Because chlamydiae are susceptible to antimicrobial therapy, their role in the development of coronary arteriosclerosis might result in a radical change in current clinical practice. However, an antibacterial treatment approach will only succeed in the presence of viable pathogens in a substantial proportion of atheromatous lesions, and mere demonstration of bacterial fragments cannot justify chemotherapy. We therefore sought to study viable bacteria in the atherosclerotic arteries to provide an indication and ethical basis for an investigational antibacterial

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Abbreviations and Acronyms

CAD = coronary artery disease
 DNA = deoxyribonucleic acid
 Ig = immunoglobulin
 PCR = polymerase chain reaction

treatment for CAD. The limitation of this approach lies in the difficulty in isolating *C. pneumoniae* by cell culture procedures. In spite of the unusually frequent occurrence of the pathogen in the respiratory tract, there are <50 continuously viable strains available worldwide. However, recent improvements in chlamydial cell culture render this approach more promising now (14). Culture results were supplemented by a nested polymerase chain reaction (PCR) method for *C. pneumoniae* genomic DNA to detect potentially viable chlamydiae nonreplicative in culture.

Methods

Coronary surgery was performed in 70 patients (60 men, 10 women; mean age 63 years, range 34 to 82) requiring elective or urgent myocardial revascularization because of total or subtotal coronary occlusion with a preserved coronary periphery. Established cardiovascular risk factors were frequent: diabetes mellitus (36%), plasma cholesterol >5 mmol/liter (57%), systolic blood pressure >140 mm Hg (70%) and tobacco smoking (24%). None of the patients reported a recent respiratory infection. Coronary samples for direct detection of *C. pneumoniae* were collected from 53 of the patients during coronary endarterectomy: after a longitudinal incision, atherosclerotic cylinders were obtained by blunt dissection of the occluding plaque from the adventitial layer. Atheromatous samples from 17 patients were obtained from restenotic bypass lesions. Immediately after harvesting, plaques were collected in a defined *C. pneumoniae* transport medium for protection of chlamydial viability (15). Perioperatively obtained sera were tested for anti-*C. pneumoniae* IgG, IgA and IgM activity in a microimmunofluorescence assay (Labsystems, Helsinki, Finland), according to the manufacturer's instructions.

Atherosclerotic tissues were cut into 0.3-cm segments and separately ground in a tissue homogenizer in culture medium at 4°C. Three segments per coronary sample were used. Each plaque suspension was divided for PCR and cell culture. The culture protocol was adapted from a recently described procedure (14). Briefly, plaque suspensions were centrifuged (1,000 g, 35°C, 45 min) onto HEP-2 host cell monolayers in multiwell tissue culture plates. Supernatants were removed, and the cultures were incubated for 3 days at 35°C, 5% carbon dioxide, in serum-free isolation medium (Eagle's minimal essential medium, nonessential amino acids, 2 mmol/liter glutamine; all from Gibco/BRL, Eggenstein, Germany), supplemented with 1 µg/ml cycloheximide (Sigma) in at least 10 serial passages. Chlamydial growth was identified by immunofluorescence

Table 1. Origin of Primer Pairs Used for PCR Analysis of Viable Cardiovascular *C. pneumoniae* Isolates

Gene	PCR Primer Pair	Amplicon Size (bp)	Study (ref no.)
53-kd protein	53-1/2	499	Kubota (20)
76-kd protein	17/1697	1681	Perez et al. (21)
16S rRNA	CpnA/B	465	Gaydos et al. (22)
<i>dnaK</i>	431/934	504	Kornak et al. (23)
<i>waaA (kdtA)</i>	184/1049*	866	Essig et al. (24)
<i>waaA (kdtA)</i>	781/1279	499	Present study

*Specific at genus level, all other primers specific at species level. bp = base pair; PCR = polymerase chain reaction; ref = reference.

microscopy: inclusions containing replicating *C. pneumoniae* were stained with fluorescein isothiocyanate-conjugated *C. pneumoniae*-specific mouse monoclonal antibody, according to the manufacturer's instructions (Cellabs, Sydney, Australia). Viable strains were continually propagated.

C. pneumoniae DNA was detected by a nested PCR method recently evaluated for carotid atherectomy tissues (16). Before amplification, DNA was purified by proteinase K digestion and cetyltrimethylammonium bromide treatment (17). Amplification relied on the species-specific HL-1/HR-1 primer pair, which amplifies a 438-base pair (bp) *C. pneumoniae* target sequence of unknown function (18), and on the nested IN-1/2 primer pair, which yields a 128-bp product (16). For confirmation and enhancement of sensitivity, nonradioactive DNA hybridization was performed using, as the probe, oligonucleotide HM-1 (18), 3'-labeled with digoxigenin-ddUTP (Boehringer GmbH, Mannheim, Germany). Strategies to prevent false positive results from contamination vigorously adhered to recent guidelines (19). For control purposes, seven coronary arteries as well as 10 segments from the aortic wall, without macroscopic evidence of atherosclerosis (13 men and 4 women; mean age 49 years, range 24 to 73), were available for PCR testing and were examined as described earlier. These samples were obtained at autopsy or from explanted hearts. The rates of chlamydial presence in atherosclerotic and healthy samples were compared using the chi-square test. The presence of chlamydiae in the coronary endarterectomy samples and in the restenotic bypass samples was compared in the same manner.

Strains isolated from vascular tissue might differ from the common respiratory isolates. For this reason and for confirmation of the identification, cardiovascular isolates were compared with the respiratory reference strains CWL-029 (American Type Culture Collection VR 1310) and MUL-1 (14) by DNA amplification involving four diagnostic PCR primer pairs known to specifically identify *C. pneumoniae* (Table 1) (20-23). Published sequence variations within the chlamydial lipopolysaccharide biosynthesis gene *waaA (kdtA)* were used to derive genus-specific (184/1049) (24) and species-specific (781/1279) primers. Sequences of the 781/1279 primers are 5'TG-GCTTCCTGTAGTGCAGAA3' and 5'ATGCTCTCCATG-TACGGTCA3', respectively. Mobility of amplification products was determined by agarose-gel electrophoresis. For

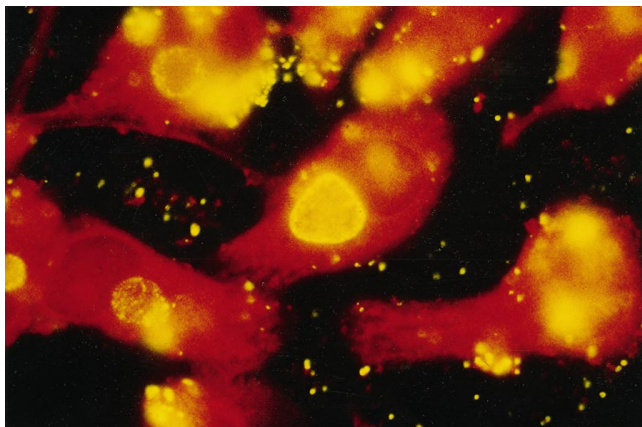


Figure 1. Immunofluorescence stain reveals infection of HEp-2 host cells with replicating *C. pneumoniae* isolated from the occluded coronary artery of a 62-year old man (passage 15 after primary isolation). Multiple inclusions in the host cells are characteristic of *C. pneumoniae*. This cardiovascular strain is morphologically identical to the common respiratory isolates.

further examination of possible differences between cardiovascular and respiratory strains, all PCR products from CWL-029 and the cardiovascular isolates CV-1 and CV-2 were purified and cloned into vector pCR2.1 (Invitrogen, Leek, The Netherlands). DNA sequences were then determined on both

strands of the clones by standard DNA sequencing methodology.

Results

Replicating *C. pneumoniae* isolates were recovered by cell culture from the occluded coronary arteries of 11 (16%) of 70 patients, indicating that viable chlamydia commonly thrive in atherosclerotic human arteries. Six isolates could be permanently propagated by serial subcultures. Isolates were identified by immunofluorescence in the second to fifth passages. Isolation of the strains was successful in all three arterial segments examined for six patients and in two segments for three patients. In two patients, one segment only yielded a viable isolate. Strains were consecutively designated CV-1 to CV-11. An immunofluorescence stain of CV-1 is shown in Figure 1.

The nested PCR protocol yielded positive results in the arterial samples of 21 (30%) of 70 patients (Table 2). All culture-positive patients were PCR-positive. Nine of the patients with a parallel positive culture result had positive PCR results in all three separately examined coronary artery segments; two were positive in two segments. Of the 10 patients without culturally retrievable chlamydiae, seven were positive for chlamydial DNA in three segments, and three were positive

Table 2. Clinical and Serologic Characteristics of 21 Patients With a Positive Result by Polymerase Chain Reaction for *C. pneumoniae* Genomic Deoxyribonucleic Acid in Their Occluded Coronary Arteries Do Not Aid in Identifying Individual Endovascular Infection

Pt No./ Gender	Age (yr)	Coronary Sample	Established Cardiovascular Risk Factors	Viable <i>C. pneumoniae</i> Strain Isolated	MIF-IgG Titer	MIF-IgA Titer
1/M	62	CEA	SBP, CHOL, TSM	CV-1	64	32
2/F	62	BYP	SBP, CHOL		64	< 16
3/F	66	CEA	CHOL, TSM	CV-2	16	< 16
4/M	55	CEA	TSM		128	64
5/M	52	CEA	SBP, CHOL, DM, TSM		32	< 16
6/M	68	CEA	SBP, CHOL	CV-3	64	32
7/F	57	BYP	SBP, CHOL		256	< 16
8/M	68	CEA	CHOL, DM	CV-4	16	< 16
9/M	67	CEA	SBP, CHOL, DM	CV-5	2,048*	256*
10/M	68	BYP	SBP, CHOL, DM	CV-6	512*	< 16
11/M	72	CEA	SBP		512*	64
12/M	67	CEA	—		256	16
13/M	71	CEA	CHOL		64	32
14/M	60	CEA	SBP		64	< 16
15/M	79	CEA	SBP, DM		16	< 16
16/M	69	BYP	SBP, CHOL, DM		32	< 16
17/M	54	CEA	SBP, CHOL, TSM	CV-7†	64	< 16
18/M	65	CEA	SBP, CHOL, DM	CV-8†	32	< 16
19/F	60	CEA	—	CV-9†	512*	< 16
20/M	63	BYP	SBP, DM	CV-10†	256	< 16
21/F	49	BYP	SBP, CHOL	CV-11†	512*	< 16

*Significantly elevated titer consistent with acute infection according to common criteria. †Strain lost viability in the cell culture system during subcultures. SBP = systolic blood pressure > 140 mm Hg; BYP = atheromatous lesion from restenotic coronary bypass; CEA = coronary endarterectomy sample; CHOL = plasma cholesterol > 5 mmol/liter; DM = diabetes mellitus; F = female; IgA = immunoglobulin A; IgG = immunoglobulin G; M = male; MIF = microimmunofluorescence assay; Pt = patient; TSM = tobacco smoking.

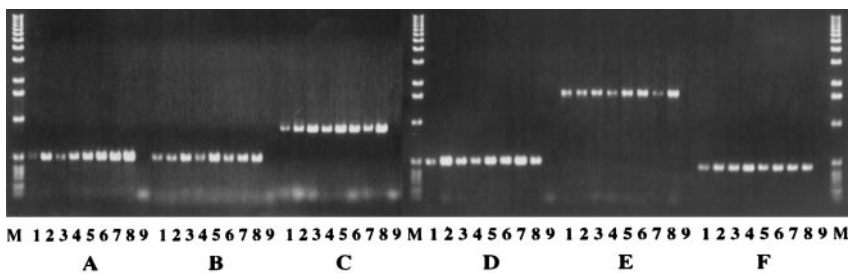


Figure 2. Polymerase chain reaction products generated with *C. pneumoniae* reference strains CWL-029 (lane 1) and MUL-1 (lane 2) and six cardiovascular isolates (lanes 3 to 8). Primer pairs 53· 1/2 for the 53-kd protein gene were applied to panel A, 781/1279 and 184/1049 for *waaA* to panels B and C, respectively, 431/934 for *dnaK* to panel D, 17/1697 for the 76-kd protein gene to panel E and CpnA/CpnB for 16S rRNA to panel F. Lane 9 contains the negative control; the marker lanes (M) contain the 1-kb ladder (Gibco/BRL). Amplification products of pulmonary and cardiovascular isolates do not differ in electrophoretic mobility, indicating that the common respiratory strains gain access to the systemic circulation and the sites of coronary arteriosclerosis. Further genetic analysis is needed to detect possible specific characteristics of vascular strains that may be linked to vascular pathology.

in two segments. Positive results from any given single segment were reproducible. No positive results were obtained from the 17 control samples without macroscopically evident atherosclerosis. This difference in chlamydial DNA detection results between atherosclerotic samples and healthy control material was significant by the chi-square test ($p \leq 0.01$). Endarterectomy samples as well as restenotic bypass lesions harbored *C. pneumoniae*: 15 (28%) of 53 endarterectomy and 6 (35%) of 17 restenotic bypass samples were PCR-positive (Table 2). This difference in chlamydial DNA detection between endarterectomy and restenotic bypass samples was not significant by the chi-square test.

Serology was not useful in identifying patients with proven endovascular *C. pneumoniae* infection. Only six of the 21 infected patients had significantly elevated IgG titers (≥ 512) consistent with recent infection, according to generally accepted microimmunofluorescence test interpretation criteria (Table 2) (2). In contrast, such elevated IgG titers were also found in six patients without detectable chlamydiae. Sixty-two (89%) of 70 patients were seropositive for chlamydial IgG, including all those with a positive direct detection result. Immunoglobulin A seropositivity, which is considered related to short- or long-term chlamydial infection, was 46%: 7 (33%) of 21 of the infected patients and 25 (51%) of 49 of those without verified infection were IgA-positive. Firm criteria for interpretation of antichlamydial IgA are lacking, but distinctly elevated IgA titers (≥ 256) occurred in one patient with endovascular infection and in two without infection. Specific anti-*C. pneumoniae* IgM was not detected. Clinical characteristics did not aid in differentiation of the patient groups, either. The established cardiovascular risk factors were similarly distributed in patients with and without verified coronary artery infection. Interestingly, patients with a positive direct detection result did not report a recent respiratory infection, nor did their initial clinical investigation reveal signs of infection. Table 2 shows microimmunofluorescence titers and clinical characteristics of patients with a positive direct detection result.

DNA amplification and sequence analysis permitted unequivocal identification of *C. pneumoniae* strains isolated from atheromatous plaques and suggested high conservation among the genes for the 53- and 76-kd proteins, as well as for *dnaK*, *waaA* (*kdtA*) and 16S rRNA. As shown in Figure 2, the continuously viable vascular strains invariably yielded amplification products of the predicted size in each experiment, identical to those obtained for the respiratory reference strains. Sequence analysis of PCR products derived from the reference strain CWL-029 and cardiovascular strains CV-1 and CV-2 revealed 100% sequence identity (data not shown). Therefore, vascular and respiratory strains could not be distinguished.

Discussion

Presence of viable chlamydiae in atheromata. Coronary artery disease has an infectious component: a significant proportion of coronary arteries occluded by atheromata harbor viable bacteria. We have culturally isolated replicating *C. pneumoniae* from 11 (16%) of 70 atherosclerotic samples obtained during myocardial revascularization. The PCR results indicate that true chlamydial infection in CAD may even be more frequent: genomic *C. pneumoniae* DNA was detected in 30% of atherectomy tissues. This finding can also be considered as evidence for the presence of viable pathogens; extrinsic DNA is usually rapidly degraded by restriction endonucleases. There was no evidence of *C. pneumoniae* in 17 nonatherosclerotic control samples. These results imply a chlamydial role in the pathogenesis of CAD, but even the endovascular presence of multiplying bacteria is not final proof of their etiologic role in coronary arteriosclerosis. In contrast, purely commensal existence of replicative bacteria in inflamed but otherwise sterile tissue is not very plausible.

Respiratory epithelium is the primary target of *C. pneumoniae*. How the chlamydiae gain access to the arteries is unclear, but infected monocytes/macrophages are potential vectors for dissemination (13). A preliminary analysis yielded

no obvious differences between reference strains from the respiratory tract and the newly recovered cardiovascular isolates with regard to size and sequence of six different PCR products. Thus, it is possible that the common respiratory strains, known to infect virtually everyone repeatedly during their lifetime, gain access to the systemic circulation. However, a more detailed analysis of the now-available cardiovascular isolates will reveal whether they have strain-specific characteristics in their genetic background that may be linked to cardiovascular pathology.

Previous studies. Attempts to directly detect endovascular chlamydiae have produced rather conflicting results, with positivity rates varying between 2% and 100%; healthy arteries did not contain chlamydiae (11-13,25,26). Immunostaining usually produced the highest positivity rates. Thus, immunofluorescence staining revealed 79% of coronary atheromata to contain structures conforming with chlamydial inclusions (25). However, a potentially decreased specificity of those immunologic detection techniques, when applied to atheromatous lesions, has been considered a problem (27). This is avoided by use of DNA amplification or cultural isolation, but only isolation can unequivocally demonstrate chlamydial viability—the prerequisite for successful antimicrobial treatment. The recent improvements of culture conditions (14) made the recovery of viable chlamydiae from a substantial proportion of plaques in the present study now possible. Notorious difficulties to recover *C. pneumoniae* have contributed to its late discovery in 1986 (1). Previous attempts of chlamydial cell culture isolation from the coronary arteries have been futile, with the exception of a single strain recently reported (12,26,28). It is not surprising that DNA amplification yielded a higher positivity rate than did culturing in our study. A clinically relevant two- to threefold enhanced sensitivity of PCR is known from respiratory samples (3). In spite of the use of optimized cell culture protocols, the attempt to recover primary isolates is commonly unsuccessful. Whether this is due to the artificial environment provided by the isolation system or the bacteria remaining in a viable but nonreplicative state of persistence is not known. The distribution of chlamydiae in the coronary arteries is apparently uneven, as we were not able to detect chlamydiae in every single fragment from an infected plaque. Therefore, the rate of vessels actually infected may even be higher than that found in our study. As other arterial tissues can be affected, too, the vascular presence of chlamydiae appears to be a generalized phenomenon of atherosclerotic diseases not limited to the coronary arteries (16,29).

Antimicrobial therapy and CAD. The endovascular presence of viable bacteria provides an indication and the ethical base required for investigational studies of antimicrobial therapy in CAD. Interestingly, chlamydiae are recovered from primary atheromatous lesions as well as from secondary restenotic bypass lesions at a similar rate (28% vs. 35%). Thus, *C. pneumoniae* may also be involved in the inflammatory processes leading to bypass restenosis. Patients undergoing coronary artery bypass graft surgery might comprise a group in which a potential benefit of antimicrobial therapy might be-

come evident within a limited period. Macrolide antibiotics are probably suitable, as respiratory *C. pneumoniae* strains are susceptible to them (30), but dosages and duration of therapy have not yet been defined. Recovery of the vascular isolates now permits us to conduct antimicrobial susceptibility tests with the potentially atherogenetically relevant strains. Some preliminary data indicating potential benefit of antimicrobial therapy in severe CAD are available (31,32), but much more extensive investigations are required before any conclusion regarding possible advantages of antichlamydial treatment in CAD can be made. Currently we do not even know whether chlamydiae exist in a continuously replicative state susceptible to antimicrobial therapy in the atherosclerotic plaques, or whether they can enter a persistent state of limited replication resulting in a very limited susceptibility to antibacterial agents. Because neither microimmunofluorescence serology nor clinical characteristics could identify individual patients with endovascular infection, we are lacking a valid variable to predict the risk of coronary artery infection. Diagnostic procedures that depend on coronary atherectomy samples are obviously too late in the course of the disease, and the development of criteria to specify the individual risk of endovascular chlamydial infection remains a diagnostic challenge.

Conclusions. Atherosclerosis shares many attributes with chronic inflammatory disease. Raised expression of adhesion molecules and cytokines, influx and proliferation of smooth muscle cells and fibroblasts and transendothelial migration and accumulation of macrophages play key roles in atherogenesis (10,33). These pathologic alterations within the vascular wall are believed to be initiated by an as yet undefined vascular injury. Initiation of the atherosclerotic lesion as well as promotion of preexisting vascular damage by chlamydial infection is certainly conceivable, but the final experimental evidence for an etiologic role in vascular pathology definitely requires the establishment of *C. pneumoniae* infection (and its subsequent eradication) in an animal model—a difficult effort, as the pathogenicity appears naturally limited to humans, although some progress has been reported (34). Success of antibacterial treatment studies might provide empiric evidence for an etiologic role of *C. pneumoniae* in the progression of coronary arteriosclerosis. However, fulfillment of Koch's postulates or their molecular correlates (35) for *C. pneumoniae* and coronary arteriosclerosis cannot be expected over a short course of time. The contribution of chlamydiae to the development of atherosclerosis is a fascinating hypothesis that may initiate a radical change in clinical practice for one of the leading causes of death—CAD.

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