Coagulation and inflammation in diabetes mellitus

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More fibrosis and thrombotic complications but similar expression patterns of markers for coagulation and inflammation in symptomatic plaques from patients with type 2 diabetes

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Abstract

Objective: One of the possible pathological mechanisms behind the increased vascular injury in diabetes mellitus type 2 (DM2) is the formation of advanced glycation endproducts (AGEs). The aim of this study was to investigate whether the presence of AGEs and specific markers for coagulation and inflammation in symptomatic atherosclerotic plaques from DM2 patients differs from plaques from non-diabetics.

Methods and results: Carotid atherectomies were obtained from DM2 patients (n=11) and controls without DM2 matched for age and other cardiovascular risk factors (n=12) who were treated for symptomatic carotid artery stenosis. Plaques were graded according to AHA classification of lesions. More fibrosis and more thrombotic complications (p= 0.007) were observed in carotid atherectomies from DM2 patients. Percentages of immunostained smooth muscle cells and macrophages in the lesions, quantified planimetrically, did not differ between the two groups. No differences were found in the immunostaining for T cells, tissue factor (TF), endothelial protein C receptor (EPCR), nuclear factor κB and the AGE carboxymethyllysine.

Conclusions: These findings underline that DM2 is associated with increased plaque complications; however a local changed presence of AGEs, TF and EPCR seems not to be involved in this end stage of atherosclerosis.
Introduction

Diabetes mellitus type 2 is a risk factor for cardiovascular disease attributable to an accelerated process of atherosclerosis. An increasing body of evidence indicates that the presence of hyperglycaemia is the primary causal factor for vascular complications in diabetic patients (1-3). An important mediator of hyperglycaemia-induced vascular injury may be the formation of advanced glycation endproducts (AGEs) (4; 5). AGEs are the result of nonenzymatic glycation and glycoxidation during normal aging. Their production is increased during hyperglycaemia. The presence of AGEs and its binding to specific receptors can lead to cellular dysfunction and changed expression of coagulation factors, which could play a role in diabetic atherosclerosis (6-9).

Tissue factor (TF) is a key player in initiating the activation of the coagulation cascade and is thought to be involved in the development of atherosclerosis and its thrombotic complications (10; 11). TF is found on the cell membrane of various cell types. Increased expression of TF has been reported in different animal models of diabetes mellitus (12; 13). Studies in patients with type 2 diabetes reported increased expression of TF on microparticles and leucocytes (8; 14). One of the possible mechanisms of this diabetes-related increase in TF expression could be the binding of AGEs to specific receptors (8; 9; 12). A role for increased TF expression has been suggested in the greater risk for cardiovascular complications in diabetic patients (15).

The endothelial cell protein C receptor (EPCR) plays a major role in the activation of the protein C anticoagulant pathway, which is an important mechanism in down-regulating thrombus formation. It has been suggested that decreased expression of EPCR plays a role in the atherosclerotic process (16). Several studies of patients with DM2 have reported a possible relation between hyperglycaemia and down-regulation of the protein C system (7; 17-19). However it is not known whether the expression of EPCR is changed in diabetic vessels.

One of the signal transduction pathways that could play a role in the changed expression of coagulation factors by AGEs is the NF-κB pathway. NF-κB plays a central role in immune and inflammatory reactions. The usually transient NF-κB dependent gene expression is exaggerated in pathological situations. NF-κB activation has been observed in atherosclerotic lesions (20). There is evidence that NF-κB activation is increased during hyperglycaemia (21) (22) which may be
involved in changed expression of proteins in diabetes mellitus type 2. Our hypothesis is that expression of TF and EPCR might be changed in diabetic atherosclerosis by an increased presence of AGEs. To test this hypothesis we have immunohistochemically analyzed the presence and tissue localization of TF, EPCR, NF-κB and Ne-(carboxymethyl)lysine (CML), which is reported to be a major AGE (23), in carotid endarterectomies from DM2 patients and matched control patients with symptomatic carotid stenosis.

**Patients and Methods**

**Patient group**

For this study, a database was used that contained paraffin-embedded specimens of various arteries and veins from 244 patients who consecutively underwent peripheral vascular surgery at the Academic Medical Center (AMC), Amsterdam, The Netherlands, between 1994 and 1998. To use this database to compare specimens from patients with DM2 and controls, a nested case-control study design was performed. First, a selection was made of all the vascular specimens of patients who had undergone carotid atherectomy because of symptomatic arterial stenosis. Second, carotid atherectomy specimens were selected from a subpopulation of patients with known DM2 at the time of intervention, treated with insulin and/or oral anti-diabetics (n=11). In addition, a matched control group (n=12) was selected, matched for coronary risk factors such as age, known hypertension (treated or known with hypertension (blood pressure > 160/90 mmHg)), hypercholesterolemia (treated or known with hypercholesterolemia (total cholesterol > 6 mmol/L)) and smoking. For group characteristics, see Table 1. Informed consent was obtained before to surgery, and the study was approved by the local ethical committee of the AMC.

**Tissue processing and histomorphology**

Carotid atherectomies were obtained and immediately fixed in 4% buffered formalin after surgical removal. Tissues were routinely processed for paraffin embedding, and sections were serially cut at 5μm from each specimen. Per atherectomy sample one to three sites were available for testing (total control sites, n= 17; total diabetes sites, n= 13). To evaluate the morphology of atherosclerotic plaques, one section was stained with hematoxylin & eosin and one with elastin van Gieson. The severity of plaque formation was graded
Table 1. Patients' characteristics.

<table>
<thead>
<tr>
<th></th>
<th>Patients without DM2 (n=12)</th>
<th>Patients with DM2 (n=11)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>69.5 ± 2.2</td>
<td>69.8 ± 2.7</td>
<td>0.471</td>
</tr>
<tr>
<td>Men (n)</td>
<td>9 (75%)</td>
<td>7 (64%)</td>
<td>0.554</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>109.4 ± 4.0</td>
<td>173.6 ± 19.3</td>
<td>0.004</td>
</tr>
<tr>
<td>Hypercholesterolemia, statin treatment (n)</td>
<td>1 (8%)</td>
<td>2 (18%)</td>
<td>0.732</td>
</tr>
<tr>
<td>Hypercholesterolemia, no treatment (n)</td>
<td>4 (33%)</td>
<td>4 (36%)</td>
<td>0.732</td>
</tr>
<tr>
<td>Hypertension, treatment (n)</td>
<td>8 (67%)</td>
<td>8 (73%)</td>
<td>0.868</td>
</tr>
<tr>
<td>Hypertension, no treatment (n)</td>
<td>2 (17%)</td>
<td>1 (9%)</td>
<td>0.865</td>
</tr>
<tr>
<td>Smoking (n)</td>
<td>3 (25%)</td>
<td>3 (27%)</td>
<td>0.951</td>
</tr>
<tr>
<td>Stopped smoking (n)</td>
<td>4 (33%)</td>
<td>3 (27%)</td>
<td>0.899</td>
</tr>
</tbody>
</table>

according the American Heart Association (AHA) classification: type I lesion, intimal thickening with an increase in macrophages and formation of scattered macrophage foam cells; type II: fatty streak consisting of layers of macrophage foam cells and lipid-laden smooth muscle cells; type III: preatheroma, potentially symptom-producing; type IV: atheroma with a more disruptive core of extracellular lipid; type Va: fibroatheroma, lipid core containing thick layers of fibrous connective tissue; type Vb: largely calcified plaque; type Vc: plaque consists mainly of fibrous connective tissue with little or no accumulated lipid or calcium; type VI lesion: complicated plaque with fissure, hematoma, and thrombus (24). The presence of thrombus was identified on the basis of the presence of platelet aggregates and erythrocytes, with or without areas or layers of granulocytes, and in continuity with plaque material. Thrombus may also show ingrowth of smooth muscle cells, indicating thrombus organisation. Adjacent serial sections were mounted for immunostaining.

Immunohistochemistry
Sections were subjected to immunohistochemistry using cell specific mouse monoclonal antibodies against vascular SMCs (anti-α-actin), macrophages (anti-CD68), endothelial cells (anti-vWF), EPCR, TF, CML, NF-κB (recognizing total p65 protein), the activated form of NF-κB (selectively recognizing the anti-p65 subunit overlapping the nuclear location signal) and rabbit monoclonal antibodies against T-lymphocytes (anti-CD3) (Table 2). Before immunostaining with anti-EPCR, anti-activated NF-κB, anti-CD68, anti-CD3 and anti-α-actin the sections were pretreated with 10 mmol/l citric buffer (pH 6.0). Before immunostaining with anti-vWF and anti-CML, the sections were pretreated with pepsin. A streptavidin-biotin complex
method was applied and immunoreactivity was visualized with either diaminobenzidine or 3-amino 9-ethyl carbazole solution. Sections stained with antibodies against vWF, α-actin, CD3 and CD68 were counterstained with haematoxylin. Positive controls were according to literature kidney tissue (anti-TF), stomach epithelium (anti-NF-κB), larger vessel endothelium (anti-EPCR) and atherosclerotic plaque (anti-CML). Staining with murine monoclonal IgG1 antibodies (DAKO A/S, Denmark) or mouse serum was used as a negative control. Rabbit IgG was used as a negative control for anti-CD3.

Table 2. Antibody Characteristics

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Monoclonal</th>
<th>Specificity</th>
<th>Concentration</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-α-actin smooth muscle</td>
<td>Mouse</td>
<td>Vascular smooth muscle cells</td>
<td>0.35 μg/ml</td>
<td>Dako, Glostrup Denmark (1D4)</td>
</tr>
<tr>
<td>Anti-CD3</td>
<td>Rabbit (polyclonal)</td>
<td>T-lymphocytes</td>
<td>2.5 μg/ml</td>
<td>Dako</td>
</tr>
<tr>
<td>Anti-CD68</td>
<td>Mouse</td>
<td>Macrophages</td>
<td>3.6 μg/ml</td>
<td>Dako (PG-M1)</td>
</tr>
<tr>
<td>Anti-vWF</td>
<td>Mouse</td>
<td>Von Willebrand Factor on endothelial cells</td>
<td>4.4 μg/ml</td>
<td>Dako (F8/86)</td>
</tr>
<tr>
<td>Anti-CML</td>
<td>Mouse</td>
<td>Ne-(carboxymethyl) lysine (AGE product)</td>
<td>1.3 μg/ml</td>
<td>Dr. CG Schalkwijk, Free University Academic Hospital, The Netherlands (34)</td>
</tr>
<tr>
<td>Anti-TF</td>
<td>Mouse</td>
<td>Tissue factor</td>
<td>10 μg/ml</td>
<td>American Diagnostica, Stamford, Connecticut (4509) HEPCR (1489)</td>
</tr>
<tr>
<td>Anti-EPCR</td>
<td>Mouse</td>
<td>Endothelial protein C receptor</td>
<td>1.5 μg/ml</td>
<td>Dr. CT Eisman, University of Oklahoma Health Sciences Center, USA (35)</td>
</tr>
<tr>
<td>Anti-NFκB p65</td>
<td>Mouse</td>
<td>Total p65, activated and non-activated form NFκB</td>
<td>20 μg/ml</td>
<td>Santa Cruz Biotechnology, Santa Cruz, California (sc-8008) Chemicon International, Temecula, CA (MAB3026)</td>
</tr>
<tr>
<td>Anti-NFκB p65</td>
<td>Mouse</td>
<td>Nuclear location signal of p65, activated form of NFκB</td>
<td>10 μg/ml</td>
<td></td>
</tr>
</tbody>
</table>

Morphometric analysis

Results of anti-α-actin and anti-CD68 were planimetrically quantified using image analysis software (Image Pro Plus, Media Cybernetics, Inc., Silver Spring, MD, USA) on a personal computer connected with a video-mounted microscope. The total tissue area of each immunostained tissue section was outlined manually on the video screen and measured. Tissue area of immunopositive stained areas in
the section were measured automatically using gray scale detection with a fixed threshold. Subsequently, SMCs and macrophage areas were calculated as percentages of the total tissue area.

For evaluation of CD3, NF-κB, and TF immunostaining, a semiquantitative score approach was chosen using the following criteria: 0: no staining; 1: < 10% plaque tissue positive; 2: 10-50% plaque tissue positive; 3: > 50% plaque tissue positive. Anti-CML staining was evaluated for immunoreactivity in SMCs, macrophages, and endothelial cells separated, in combination with the cell-specific antibodies for actin, CD68, and vWF, using serial sections. Anti-EPCR staining was evaluated in combination with the anti-vWF stained section only. For each cell type, we used the following semiquantitative score: 0: no staining; 1: only scarce cells positive; 2: circa 50% of cells positive; 3: most cells in section positive. Observers were blinded to the clinical status of the patients.

Statistical analysis

Results are expressed as mean ± SEM for continuous variables. Categorical data are expressed as medians with 25-75 quartiles. For comparison between continuous variables of interest, Student’s t test was used. The association between categorical variables was assessed using the Mann-Whitney test or the Pearson Chi-square when appropriate. P values of < 0.05 are considered statistically significant.

Results

Patient group

For patient characteristics see Table 1. There were no significant differences between diabetic patients and controls with regard to age, gender, hypercholesterolemia, hypertension and smoking. The average serum glucose levels of the diabetic patients (173.6 ± 19.3 mg/dL) were significantly higher than those of the control patients (109.4 ± 4.0 mg/dL) (p< 0.004).

Morphological features of atherectomies

The occurrence of different lesion types was compared between control and diabetic atherectomies. To make comparison possible between the relatively low frequencies per lesion type, lesion types were grouped as either type III, IV and Va lesions or type Vc and VI lesion. Carotid atherectomies from DM2 patients showed significantly more fibrotic lesions (type Vc lesions) and more thrombotic
complications (type VI lesions) (for typical example, see Figure 1) compared to control lesions (85% vs. 31%) (p = 0.007), (Figure 2). No differences were observed in the amount of SMCs, macrophages and T cells between atherectomies from DM2 patients and controls (Table 3 and 4).

Immunohistochemistry of TF, EPCR, NF-κB and CML
No differences were observed in the presence of TF, EPCR, NF-κB and CML staining in lesions from DM2 patients and controls (Table 4). Staining for CML varied widely from nearly absent to strong throughout the different plaques. However, no differences were found between DM2 patients and controls. CML was located in SMCs, macrophages and in the extracellular matrix (Figure 3C and

Figure 1. Example of atherosclerotic plaque with thrombus (type VI lesion) from patient with DM2. Tissue is stained with haematoxylin & eosin (magnification 40 X).

Figure 2. Histological grading of plaque morphology according to the AHA classification in diabetic and control patients. Significant more type Vc and VI lesions were observed in diabetic patients as compared to controls (p=0.007) (Pearson Chi-square test).

Table 3. Morphometric analysis of SMC and macrophage immunostaining in diabetic patients and control patients. Data are presented as means ± SEM.

<table>
<thead>
<tr>
<th>Immunostaining</th>
<th>Controls</th>
<th>Diabetic patients</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smooth muscle cells</td>
<td>0.08 (± 0.019)</td>
<td>0.05 (± 0.007)</td>
<td>0.21</td>
</tr>
<tr>
<td>Macrophages</td>
<td>0.03 (± 0.003)</td>
<td>0.02 (± 0.005)</td>
<td>0.22</td>
</tr>
</tbody>
</table>
Diabetic atherectomy

Table 4. Semi quantitative analysis of TF, NF-κB, CML, EPCR and T cells in diabetic and control patients. Data are presented as medians with 25-75 quartiles

<table>
<thead>
<tr>
<th>Immunostaining</th>
<th>Controls</th>
<th>DM2 patients</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tissue factor</td>
<td>2.0 (2.0, 3.0)</td>
<td>2.0 (1.0, 2.0)</td>
<td>0.37</td>
</tr>
<tr>
<td>NF-κB</td>
<td>1.0 (0.5, 2.0)</td>
<td>1.0 (0.0, 2.0)</td>
<td>0.71</td>
</tr>
<tr>
<td>Activated NF-κB</td>
<td>1.0 (1.0, 1.0)</td>
<td>1.0 (1.0, 1.0)</td>
<td>0.76</td>
</tr>
<tr>
<td>CML smooth muscle cells in intima</td>
<td>1.0 (1.0, 1.8)</td>
<td>1.0 (1.0, 1.8)</td>
<td>0.95</td>
</tr>
<tr>
<td>CML smooth muscle cells in media</td>
<td>1.0 (1.0, 1.0)</td>
<td>1.0 (1.0, 1.5)</td>
<td>0.14</td>
</tr>
<tr>
<td>CML extra cellular matrix</td>
<td>0.5 (0.0, 1.0)</td>
<td>1.0 (0.0, 1.0)</td>
<td>0.23</td>
</tr>
<tr>
<td>CML endothelium</td>
<td>1.0 (0.0, 1.0)</td>
<td>1.0 (0.0, 1.0)</td>
<td>1.00</td>
</tr>
<tr>
<td>EPCR endothelium plaque</td>
<td>1.0 (1.0, 2.0)</td>
<td>1.0 (1.0, 2.0)</td>
<td>0.91</td>
</tr>
<tr>
<td>EPCR microvascular endothelium</td>
<td>0.0 (0.0, 0.5)</td>
<td>0.0 (0.0, 1.0)</td>
<td>0.78</td>
</tr>
<tr>
<td>T cells</td>
<td>2.0 (1.0, 2.0)</td>
<td>2.0 (1.0, 2.5)</td>
<td>0.83</td>
</tr>
</tbody>
</table>

Figure 3A. Carotid artery atherosclerotic plaque classified as a type Vb lesion according to AHA classification (magnification 20X). Tissue section is stained with Elastic von Gieson (collagen = red, elastin = black, cells = yellow/brown). The plaque shows extensive fibrosis (*F)*, fibrocellular area with neovascularization (FC) and a deeply located atheroma (asterisk). L = lumen. Fig 3B-D are details of boxed area in Fig 3A, immunostained in adjacent serial sections. Fig 3B. Immunostaining with anti-vWF, showing immunoreactivity of endothelial cells lining micro vessels inside the plaque (magnification 80X). Fig 3C. Anti-CML antibody showing immunostaining of endothelium of all micro vessels (magnification 80X). Fig 3D. Anti-NF-κB antibody showing immunostaining of microvascular endothelium and scattered inflammatory cells (magnification 64X).
Figure 4. Fig 4A. Carotid artery plaque type Vb lesion stained with elastic van Gieson. L = lumen; F = fibrous tissue; M = media (magnification 20X). Boxed area is at the border zone of a partly fibrosed lipid core and fibrous cap tissue (Fig 4B-D magnification 80X). Fig 4B. Immunoreactivity of TF in foam cell macrophages bordering the atheroma. Fig 4C. Cellular immunolocalization of AGEs at the same place as shown in Fig 4B. Fig 4D. Anti-NF-κB stained section showing immunostaining of foam cell macrophages and SMC in same area.

Figure 5. Fibrous plaque (Type Vb), stained with anti-EPCR antibody. Fig 5A shows immunoreactivity of scarce endothelial cells lining micro vessels inside the plaque (magnification 80X). Fig 5B shows prominent continuous immunoreactivity of arterial endothelium covering the plaque surface (magnification 55X).
Staining for TF was located in SMCs and macrophages (Figure 4B). In 87% percent of the plaques, co-localization of CML and TF in macrophages was observed, using two adjacent sections. There was no difference in the number of patients showing co-localization of CML and TF between the diabetic and nondiabetic group. EPCR staining of the atherosclerotic plaque endothelium was weakly positive in endothelium of all atherosclerotic plaques (Figure 5B). EPCR staining in microvessel endothelium ranged from absent to weak positive (Figure 5A). There were no differences in EPCR staining of the plaque endothelium or the microvessel endothelium in the DM2 group compared with the control group (Table 4). In nearly all atherectomies, only weak NF-κB-p65 staining was observed, mainly located in SMCs, macrophages, and endothelial cells (Figure 3D and 4D). To identify activation of NF-κB we used an antibody specifically against activated NF-κB (MAB3026). A weak staining pattern of activated NF-κB was observed, which was located in nuclei of SMCs, macrophages and endothelial cells (Figure 6). No difference in staining was observed between diabetic and control plaques (Table 4).

Discussion

Hyperglycaemia is considered to play a major role in diabetic cardiovascular disease. In particular, the formation of AGEs appears to be an important mediator of hyperglycemia-induced vascular injury (4; 25; 26). Several experimental studies showed that AGEs influence the production of proteins involved in the coagulation process in different cell types, which might play a role in the increased risk of thrombotic complications in diabetic patients (9) (7; 8; 27; 28). In the present
study, simultaneous tissue localization of AGEs and coagulation proteins was studied in symptomatic atherosclerotic plaques from diabetic patients and their matched controls. Our data showed that plaques from DM2 patients had a significantly higher number of fibrotic lesions and lesions with thrombotic complications with those of matched control patients, confirming the well-known observation that DM2 is associated with an increased incidence of cardiovascular complications. Despite these differences, a consistent lack of differences in immunostaining of AGEs, TF, EPCR, and NF-κB was observed.

The lack of difference in AGE staining in the present study might be explained by the symptomatic, end-stage atherosclerosis that was studied and is in agreement with earlier studies that reported that AGEs are associated with atherosclerotic lesions regardless of the presence of diabetes (29; 30). In particular, several studies showed that the degree of AGE staining correlated with the extent of the atherosclerotic changes (31; 32) in non-diabetic and diabetic patients. These findings suggest that the quantity of AGEs in atherosclerotic lesions is more related to the severity of the atherosclerosis than to the presence of hyperglycaemia.

The age of the studied patients, mean of 70 years, is a second possible explanation for the lack of increased AGEs staining found in the DM2 group. It has been observed that AGE accumulation in tissue is related to aging (30; 32; 33). It may be possible that the role of AGEs is more important in an earlier stage of the accelerated atherosclerosis in diabetes mellitus and that differences in AGEs localization are more distinct in plaques of younger diabetic patients or in less complicated plaques. This could explain why in other studies differences where observed between atherosclerotic lesions from diabetic patients and controls.

TF, the main initiator of the coagulation cascade, is widely expressed in atherosclerotic plaques and is thought to play a role in the development of acute arterial thrombosis. In agreement with earlier observations (11) we clearly observed TF in macrophages, SMCs and foam cells. However, this staining pattern was similar in control and diabetic plaques. This is in contrast to animal studies that showed that TF is increased in tissue and plaques from diabetic mice (12; 13), which is probably related to accelerated atherosclerosis and enhanced AGE formation. The latter could explain the lack of difference in TF expression in the present study. AGEs, however, still might have been involved in TF expression in both the diabetic and non-diabetic plaques, since the TF pattern co-localized
with the CML staining in almost 90% of both control and diabetic lesions. Activation of the NF-κB pathway is a possible mechanism that may contribute to the changed expression of coagulation factors by AGEs (21). But NF-κB activation may also be involved in other inflammatory atherogenic processes (20). The detection of similar staining of activated NF-κB confirms a role for activation of the NF-κB pathway in both diabetic and non-diabetic atherosclerosis. EPCR expression was found to be decreased in endothelial cells covering atherosclerotic lesions compared with the expression in endothelial cells in control arteries according to Laszik et al (16). In agreement with these findings, a weak positive staining of EPCR on endothelium covering the atherosclerotic plaque was observed in our study. Although it has been suggested that the protein C system may be down regulated in DM2 (17-19), we could not find a difference between expression of EPCR in atherectomies of patients with DM2 and controls in this study. Neither did we find a relationship between AGEs and EPCR staining. This might be attributable to other regulatory mechanisms that have an effect on EPCR expression in atherosclerosis.
In summary, we found a significantly higher number of fibrotic lesions and lesions with thrombotic complications in plaques from DM2 patients. However, we did not find evidence that this could be related to increased presence of AGEs and the changed expression of coagulation proteins in diabetic atherosclerotic lesions. It may be that in this end stage of atherosclerosis, no more local differences in the presence of these proteins can be distinguished, although they might have played a role in an earlier stage of the process. Preexisting differences in the expression of coagulation and inflammation markers may have been obscured by the advanced stage of atherosclerosis. Thus, to elucidate the question whether DM2 leads to a changed expression of inflammation and coagulation markers and whether this plays a role in the accelerated atherosclerotic process, studies of less advanced presymptomatic atherosclerotic lesions may be necessary.

Acknowledgements
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