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Autoantibodies against MDA-LDL in subjects with severe and minor atherosclerosis and healthy population controls

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Abstract

Autoantibodies against oxidized low-density lipoprotein (LDL) have been reported to be associated with atherosclerosis. However, data are not consistent.

We compared the titres of autoantibodies to malondialdehyde-modified LDL in three groups, a case group with angiographically documented severe coronary stenosis (> 80% stenosis in at least 1 vessel, $n = 47$), a hospital control group with minor stenosis on the coronary angiography (< 50% stenosis in all three major vessels, $n = 47$) and a healthy population control group with no history of coronary heart disease ($n = 49$). Age ranged from 26 to 68 years. Subjects were frequency-matched for gender distribution and storage time of the blood samples. No relevant differences in autoantibody titre between case and control groups were found. The mean autoantibody titres ($\pm$ S.D.) were 1.44 $\pm$ 1.82, 1.46 $\pm$ 1.40 and 1.62 $\pm$ 1.95 for cases, hospital controls and population controls, respectively. No correlations were found between autoantibody titre and age, number of cigarettes smoked and LDL or total cholesterol. Autoantibody titres were correlated with body mass index ($r = 0.2$) and high-density lipoprotein (HDL) ($r = -0.2$). Odds ratios (OR) were calculated by tertiles of autoantibody titres for the hospital control group and the population control group, respectively. Age-adjusted OR (95% confidence interval) for medium and high compared to low autoantibody titre were 0.76 (0.27–2.14) and 1.09 (0.39–2.95) for the comparison between cases and hospital controls and 1.09 (0.39–3.07) and 0.90 (0.32–2.56) for the comparison between cases and population controls. Adjustment for gender, body mass index, smoking habits and HDL yielded essentially the same results.

This study does not support an association between autoantibody titres to oxidized LDL and the extent of coronary stenosis.

Keywords: LDL oxidation; Atherosclerosis; Cardiovascular diseases; Autoantibodies

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1. Introduction

Studies implying that oxidative modification of low-density lipoprotein (LDL) takes place in vivo and may play an important role in atherogenesis have accumulated over recent years [1–4]. Oxidized LDL can activate endothelial cells and induce endothelial damage, thereby allowing blood elements and monocytes to enter the sub-endothelial space, which is the beginning of the atherogenic process. As a consequence of oxidation, the uptake of LDL by macrophages is accelerated and foam cells are formed. Furthermore, modified LDL is immunogenic and has the ability to induce the formation of autoantibodies [4,5]. Autoantibodies against epitopes of oxidized LDL have been found in several studies in both human [6–15] and rabbit [6,15,16] plasma and atherosclerotic lesions, however data on the relation between autoantibody titres and coronary atherosclerosis are not consistent [6–10,12,13,17–19]. Though the LDL-oxidation hypothesis is attractive in explaining the mechanism of development of atherosclerosis, direct evidence from human studies is still scarce.

To investigate the association between autoantibody titres and atherosclerosis, we studied autoantibody titres of three groups differing in levels of coronary artery disease, namely a group of patients with angiographically documented severe atherosclerosis, a group with angiographically documented minor or non-atherosclerosis and a population-based group with no history of cardiovascular disease (CVD).

2. Subjects and methods

2.1. Study population

Groups of patients and hospital controls were selected from participants in a previous hospital-based case-control study on angiographically documented CVD and plasma levels of cholesterol oxidation products. A group of population controls consisted of participants in the Dutch National Cardiovascular Disease Risk Factor Monitoring Project [20]. The hospital groups consisted of patients who had undergone a coronary angiography for suspected CVD in the period 1991 and 1992 in Rotterdam. Ineligible were those patients: over 68 years of age; with a previous bypass surgery; with a myocardial infarction (MI) in the 12 months prior to the study period; under cardiac care for more then 2.5 years; in whom more then 2 months elapsed between angiography and case selection; who had diabetes mellitus, liver, kidney or thyroid disease, or showed evidence of alcohol or drug abuse. Of the 387 patients eligible for this study, 51 refused to participate, 22 could not be contacted or were otherwise indisposed and 7 had died. From the remaining 307 patients, cases were selected on the basis of having more then 80% stenosis in at least one of the three major coronary vessels, and controls having less then 50% stenosis in all three major coronary vessels. This left 159 patients (80 cases and 79 controls) for the original study. For the study reported here, 50 cases and 48 controls were randomly selected for determination of plasma autoantibodies.

The population control group is a sample out of 36 000 participants in the Cardiovascular Disease Risk Factor Monitoring Project (1987–1991) [20]. For the original study, a random sample of men and women aged 20–59 were selected from the civil registry of three cities in the Netherlands. The study was performed at the basic health service in each city. For the study reported here, the following selection criteria were used: domiciled in Amsterdam, the Netherlands, without use of hypercholesterolaemic drugs, not under cardiac care, no heart surgery (such as bypass surgery), without a myocardial infarction. The 51 persons selected were frequency-matched for gender distribution and storage time of the blood samples to subjects of the hospital study.

2.2. Data collection

For all three groups, information on medical history, use of medication, dietary, smoking and drinking habits, occupation and family history of CVD was obtained through a questionnaire. Further data on height, weight and blood pressure were gathered. The hospital cases and controls

were seen within 2 months after angiography. Fasting venous blood samples were collected into a 10 ml EDTA vacutainer tube and the isolated plasma was stored at −80°C. For the population control group, non-fasting venous blood samples were collected in 10 ml EDTA vacutainer tubes and after centrifugation plasma was stored at −20°C. The mean storage period of blood samples was 31 ± 3 months (mean ± S.D.). Storage periods for cases and control groups were similar.

2.3. Measurement of anti-MDA-modified LDL autoantibodies

Autoantibody titres were measured by bi-site sandwich ELISA using polystyrene microtitre plates (Greiner, number 655001, Alphen a/d Rijn, The Netherlands). The microtitre plates were coated with MDA-LDL (malondialdehyde-modified LDL) as antigen (10 μg/ml, 100 μl/well) in phosphate-buffered saline (PBS) for 16 h at 4°C. Plates were washed 4 times with PBS and the residual binding sites were blocked with 1% (w/v) casein (Merck) in PBS (200 μl/well) for 2 h at room temperature. Plates were washed 4 times with PBS, 100 μl/well of diluted samples for autoantibody determination was added, and the plates were incubated for 16 h at 4°C. Five dilutions, 1:8, 1:16, 1:24, 1:32, 1:64 (v/v), in blocking buffer containing 0.05% (w/v) Tween 20 (Merck) were applied. After washing 5 times with PBS containing 0.05% (w/v) Tween 20, 100 μl/well goat-anti-human IgG-Fc fragment conjugated to horseradish peroxidase (Nordic, Tilburg, The Netherlands), diluted 1:11000 in blocking buffer containing 0.1% (w/v) casein and 0.05% (w/v) Tween 20, was added and the plates were incubated for 2 h at 37°C. Plates were washed 4 times and the peroxidase-labelled conjugate was visualized using 3,3',5,5'-tetramethylbenzidine and H_2O_2 as substrate mixture [21] (Organon Technika BV, Boxtel, The Netherlands). Each microtitre plate contained equal numbers of samples from the three study groups.

Extensively modified MDA-LDL was used and prepared essentially as described by Palinski et al. [6] by incubating 1 mg/ml LDL (prepared from pooled plasma from 5 male and 7 female healthy volunteers aged 21–35) in PBS, pH 7.4 for 4 h at 37°C with 0.1 M MDA, freshly prepared from malonaldehyde-bis-dimethylacetal (Kodak Eastman Co.). After conjugation, MDA-LDL was extensively dialysed against PBS and stored in the presence of 10% sucrose at −80°C. As additional antigen, native LDL (prepared from the above mentioned pool) was used in the assay. This LDL was protected from oxidation by addition of 10 μM EDTA and 20 μM butylated hydroxytoluene (Sigma) to PBS and stored in the presence of 10% sucrose at −80°C.

Each microtitre plate contained a dilution series (1:8, 1:16, 1:24, 1:32, 1:64 v/v) of the above-mentioned pool plasma in triplicate, which was used as a reference standard. From the response of these dilution series, a reference line for autoantibody response was constructed. Intra- and inter-assay coefficients of variation were 6.0% and 8.3%, respectively, for the reference curves. The autoantibody titre is defined as the ratio between the dilution on the reference line belonging to the autoantibody response in the sample and the original dilution of the sample and is, in general, the mean of the four autoantibody titres obtained at dilutions 1:8, 1:16, 1:24 and 1:32 (v/v). In most cases, the optical density (OD) at the 1:64 (v/v) dilution was near the background and deviated from the linear curve. This dilution was then omitted from the calculation. The slopes of the calibration curve constructed with reference standard and of the curves of the individual samples from the patient group and the control groups were not identical. About 15% of the sample curves deviated from the reference curve, indicating that the values calculated for the human samples can only be considered as indicators of autoantibody concentrations. This allows comparisons between different samples but cannot be considered as an accurate measure of the absolute autoantibody mass in each sample.

Alternatively, data are expressed as the absolute value for the ratio of autoantibody binding to MDA-LDL/native LDL (both as OD), as applied by Salonen et al. [9]. The presented value is the mean of the ratios obtained at dilutions 1:8 and 1:32, as the response to native LDL was only measured at these dilutions. Binding to native

Table 1
Baseline characteristics (mean ± S.D.)

	Cases (n = 47)	Hospital controls (n = 47)	Population controls (n = 49)
Age (years)	54.2 ± 8.9	53.5 ± 9.2	49.6 ± 7.8*,**
Body mass index (kg/m^2)	26.2 ± 3.3	25.4 ± 2.8	25.9 ± 3.4
Plasma cholesterol (mmol/l)	6.3 ± 0.9	5.9 ± 1.1	5.6 ± 1.0*
HDL-cholesterol (mmol/l)	1.1 ± 0.3	1.3 ± 0.4*	1.0 ± 0.3*,**
LDL-cholesterol (mmol/l)	4.4 ± 0.8	4.0 ± 1.0*	3.9 ± 0.9*
Plasma cholesterol/HDL	6.0 ± 1.6	4.9 ± 1.7*	6.3 ± 1.9**
Plasma triglycerides (mmol/l)	1.8 ± 0.8	1.5 ± 0.8*	1.7 ± 0.9
Systolic pressure (mmHg)	135.0 ± 19.4	134.0 ± 15.1	127.0 ± 16.5*,**
Diastolic pressure (mmHg)	84.8 ± 10.6	83.4 ± 10.9	79.3 ± 10.3*
% Male	68.1	68.1	67.3
% Smokers	25.5	27.7	38.8

*significant difference ($P < 0.05$) with cases
**significant difference ($P < 0.05$) with hospital controls

LDL, considered as a non-specific control, is defined as the ratio between the autoantibody response in the sample to native LDL (as OD) and the autoantibody response in the reference standard to native LDL (as OD).

2.4. Analytical measurements

HDL-cholesterol and triglycerides were determined as described by Sullivan et al. [22] and Warnick et al. [23]. Total-cholesterol was determined with a spectrum analyser (Abbott Laboratories, USA) with CHOD-PAP reagent (cat. no. 236691, Boehringer Mannheim). LDL-cholesterol was calculated with the Friedewald formula [24]. With this formula, triglyceride concentrations must not exceed 4.52 mmol/l [25]. Subjects who had a triglyceride level above 4.52 were excluded from further analysis (3 cases, 1 hospital control and 2 population controls).

2.5. Data analysis

Data analysis was conducted using the BMDP statistical package [26]. Basic characteristics for the three groups were compared by Student's t-test for unpaired samples ($P < 0.05$) and by the Mann-Whitney test for non-normal distributions. Pearson χ^2 analysis was applied for class variables. By means of the Pearson correlation coefficient, the associations between autoantibody titres and continuous variables in the total group were quantified.

To adjust differences between cases and controls for possible confounders, multiple linear regression was used. Stratified analysis was performed to identify confounders or effect modifiers. Odds ratios were calculated to quantify the association between autoantibody titre and coronary stenosis, the patients were divided into tertiles based on autoantibody titres in the hospital or population control group. Multiple logistic regression adjusted the odds ratio for potential confounders.

3. Results

In Table 1 the baseline characteristics of the three study groups are presented. The population control group was slightly, though significantly, younger than the two hospital groups. No differences were seen for body mass index (BMI). The hospital control group had higher HDL (high-density lipoprotein) and lower LDL and triglycerides than the cases. The population controls had lower total cholesterol, HDL, LDL and systolic and diastolic blood pressure than the cases. After adjustment for age and smoking habits, the significant difference in HDL disappeared.

Autoantibody titres between cases (1.44 ± 1.82 (mean ± S.D.)), hospital controls (1.46 ± 1.40) and population controls (1.62 ± 1.95) were not significantly different. Fig. 1 shows frequency histograms of the autoantibody titres of the three

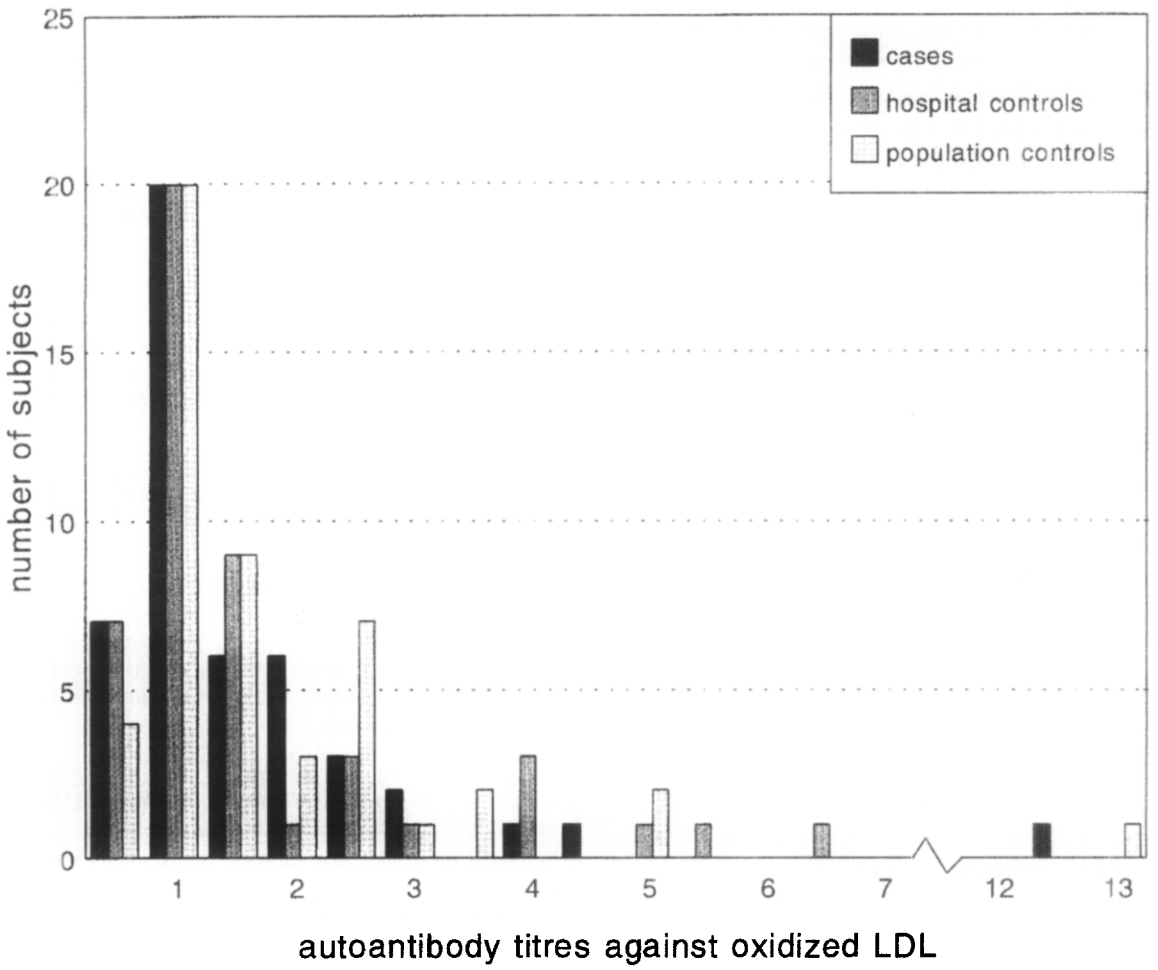

Fig. 1. Distribution of autoantibody levels against MDA-LDL among cases, hospital controls and population controls.

study groups. At the 1:8 and 1:32 dilutions, the autoantibody response to native LDL was measured. In 42 out of 143 persons, a response higher than 0.2 OD in both dilutions was found. These higher responses to native LDL were equally distributed over the three groups as determined with frequency calculations. Titres to native LDL (mean ($\pm$ S.D.)) were similar for the three groups. The OD assessed at dilution 1:8 were 0.34 $\pm$ 0.35, 0.37 $\pm$ 0.36 and 0.32 $\pm$ 0.35 and at the 1:32 dilution 0.23 $\pm$ 0.31, 0.22 $\pm$ 0.22 and 0.21 $\pm$ 0.20 for cases, hospital controls and population controls, respectively. None of the differences reached significancy.

Table 2 presents correlation coefficients for the total population between risk factors for CVD and autoantibody titres. Only BMI and HDL showed a significant relationship with autoantibodies. When LDL is being oxidized, a series of oxidative products of cholesterol are produced [27,28]. We, therefore, also assessed the relationship between cholesterol oxidation products (total oxysterols, 7α-OH cholesterol (the main oxysterol, but also an important intermediate in the bile acid synthetic pathway) and total oxysterols without 7α-OH cholesterol) and autoantibody titres in the case and the hospital control group. Correlations were -0.05, -0.02 and -0.07 for

total oxysterols, 7α-OH cholesterol and total minus 7α-OH cholesterol, respectively. None of these correlations reached significance.

Odds ratios for the risk of CVD and autoantibody titre were calculated with logistic regression analyses for tertiles of autoantibody titre for cases and hospital controls and for cases and population controls. Results are shown in Table 3a. No significant associations were observed for autoantibody titres and CVD.

Additionally, we performed the analysis on data obtained by using the calculation method proposed by Salonen et al. [9], in which autoantibody titres are expressed as the ratio titre (binding to MDA-LDL divided by binding to native LDL, both as OD). The mean (± S.D.) ratio titres for cases, hospital controls and population controls were 4.54 ± 2.96, 4.26 ± 2.72 and 4.90 ± 3.06, respectively. Comparable to the above mentioned approach, no relevant differences among the groups were found.

When assessing the correlation coefficients between the ratio titre and risk factors for CVD, none of the variables age, number of cigarettes, BMI, systolic and diastolic blood pressure, total cholesterol, HDL, LDL and triglycerides showed a significant association (Table 2).

Odds ratios calculated for tertiles of the ratio titre in the control group are presented in Table 3b. No significant association was found between cases and hospital controls and between cases and population controls. Adjustment for gender, age, smoking habits, HDL and BMI had no impact on the results.

Table 2
Correlations for all data between autoantibody titres and risk factors for CVD

Total (n = 143)	Autoantibody titre	Ratio titre*
Age	−0.01	−0.14
Number of cigarettes/day	0.06	−0.08
Body mass index	0.20**	0.09
HDL	−0.19**	−0.15
Total cholesterol	−0.08	−0.13
Triglycerides	0.03	0.02
LDL	−0.03	−0.10
Systolic pressure	0.05	0.04
Diastolic pressure	0.00	0.02

*Autoantibody titres were calculated according to the method proposed by Salonen et al. [9].
**$P < 0.05$

Table 3
Odds ratios and 95% confidence intervals (CI) for the risk of CVD in tertiles of auto antibody titres in the controlgroup, for case and hospital control groups and cases and population controls

	Age-adjusted	Multivariate adjusted*
(a) Autoantibody titre		
Cases hospital controls		
<0.76	1.0	1.0
0.76–1.17	0.76 (0.27–2.14)	0.63 (0.21–1.93)
>1.17	1.09 (0.40–2.95)	0.77 (0.26–2.27)
Cases/population controls		
<0.75	1.0	1.0
0.75–1.42	1.09 (0.39–3.07)	1.32 (0.45–3.89)
>1.42	0.90 (0.32–2.56)	1.08 (0.36–3.25)
*(b) Ratio titre***		
Cases/hospital controls		
<2.2	1.0	1.0
2.2–4.9	2.22 (0.75–6.58)	1.51 (0.46–4.95)
>4.9	2.04 (0.67–6.21)	1.60 (0.49–5.23)
Cases/population controls		
<3.0	1.0	1.0
3.0–5.9	0.98 (0.36–2.68)	0.93 (0.32–2.76)
>5.9	0.71 (0.25–2.06)	0.74 (0.25–2.22)

*Adjusted for gender, age, smoking habits, HDL, body mass index.
**Autoantibody titres were calculated according to the method proposed by Salonen et al. [9].

4. Discussion

In this study, no association between coronary heart disease and autoantibody titres was found.

It is unlikely that the absence of differences in autoantibody titres is due to flaws in the study design. Blood samples were stored at −80°C or −20°C degrees. There is no reason to assume that differences in storage temperature have affected the association, as autoantibody titres in

plasma at the different storage temperatures were within the same range. Storage time for the three groups was comparable.

As it is conceivable that dietary or life-style changes may affect LDL oxidation in vivo [29–32] and thus may alter autoantibody production, the cases and hospital controls were examined within 2 months after the angiography. Thus, dietary and life-style changes as a response to the cardiology report are restricted.

The two hospital groups were slightly older than the population group (mean age of 54 and 50 for hospital and population groups, respectively). This could have introduced bias, but no association between autoantibody titres and age was found. Further, the result could have been biased by smoking habits, since there were more smokers in the population control group. Smoking might be related to oxidative stress [33–36] and thus could increase oxidation of LDL. Yet, no difference in autoantibody titres between smokers and non-smokers was found and calculation of correlations between autoantibody titres and number of cigarettes smoked did not produce a relevant association. In addition, in a previous report we did not observe an effect of smoking on susceptibility of LDL to oxidation [36].

We compared the autoantibody titres of the cases to both a hospital and a population control group. An objective discrimination between cases and hospital controls is possible based on the angiographical data, implying as a control group a group with documented minor stenosis. However, in the control group, 64% were scored as having no stenosis, 23% had less than 10% stenosis in the 3 vessels and the mean percentage stenosis over the 3 vessels did not exceed 30%. The population control group was a selection of healthy men and women without self-reported history of CVD. However, we cannot rule out the possibility of people with unknown, clinically non-manifested CVD entering the control group.

In this study, we defined autoantibody titres in two different ways: one related to a reference line constructed from the response to a reference plasma and the other being the ratio between the response to MDA-LDL and that to native LDL. The advantage of the first method is the use of several dilutions. The autoantibody titres presented are generally the mean of the autoantibody titres obtained at four dilutions. We believe to have thus reduced the approximation error. When plotting the OD against the concentration, individual curves do not parallel each other. As pointed out by Virella et al. [19], we therefore should not use these autoantibody titres as an absolute measure, but rather as an indicator of autoantibody concentrations. The second method for expressing autoantibody titres was calculated at the 1:8 and 1:32 dilutions. Both methods were significantly correlated ($r = 0.34$, $P < 0.001$). As both ways of expressing the data generate the same result, it is justified to conclude that there is no reason to assume that our result can be ascribed to our definition of autoantibody titres.

The results of our study are consistent with reports from several groups, in which no difference in levels of antibodies were found [6,17–19], but are inconsistent with others [7,9,10,12–14]. In most studies, elevated levels of autoantibodies against epitopes of oxidized LDL are reported both in subjects with coronary heart disease and in healthy controls. Findings of Maggi et al. [10], Salonen et al. [9], Puurunen et al. [13], as well as the results of Virella et al. [18] who described higher (though not significantly higher) autoantibody titres in hyperlipidaemic persons and in subjects with minor atherosclerosis in comparison to persons with known CVD, support the idea that antibodies are not an indicator of the severity of atherosclerosis, i.e. the extent of thickening of the vessel wall, but can be used as an indicator for an active atherogenic process. In our study, no data on the change in extent of stenosis in recent years were available. We, therefore, can not exclude the possibility that subjects were in a stable state of atherogenesis. This may provide an explanation for the lack of difference in our study. Another explanation for not finding a relation with the extent of CVD is pointed out by Virella et al. [19] who suggest that different persons may have different populations of antibodies with a different affinity to antigens.

We conclude that this study does not support an association between autoantibody titres to oxidized LDL and thickening of the vessel wall. We

suggest that clinical studies focus on assessment of the relationship between autoantibody levels and development of new lesions, e.g. by measurement of intima-media thickening by ultrasound.

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