The Carotid Artery Plaque Size and Echogenicity are Related to Different Cardiovascular Risk Factors in the Elderly

The Prospective Investigation of the Vasculature in Uppsala Seniors (PIVUS) study

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Abstract Carotid plaques can be characterised by ultrasound by size and echogenicity. Both size and echogenicity are predictors of cardiovascular events. The aim of this study was to examine whether traditional risk factors and markers of inflammation and oxidation were associated with plaque size and echogenicity. Computerised analysis of carotid plaque size and echogenicity (grey scale median, GSM) were performed by ultrasound in a population-based health survey in 1,016 subjects aged 70 years (PIVUS study). Information on cardiovascular risk factors was collected, together with markers of inflammation and oxidation. Increased Framingham risk score, systolic blood pressure, higher BMI and decreased HDL, lower glutathione levels were related to echolucent plaques. Previous or present smoking was common with significantly more pack-years related to the echorich plaques. Plaque size was associated with increased Framingham risk score, systolic blood pressure, blood glucose levels, smoking, ApoB/A1 ratio, OxLDL, TNF alpha, HOMA insulin resistance, leucocyte count, decreased BCD-LDL and low levels of l-selectin. Low HDL, increased BMI and decreased glutathione levels were associated with the echolucency of carotid plaques, implying metabolic factors to play a role for plaque composition. Markers of inflammation were related to plaque size alone, implying inflammation to be predominantly associated with the amount of atherosclerosis. These results suggest that plaque size and echogenicity are influenced by different risk factors.

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Introduction

Plaque rupture is believed to be the precipitating event in the major clinical manifestations of atherosclerosis, e.g. myocardial and cerebral infarction. The vulnerable plaque is described as containing a large lipid-rich core, a thin fibrous cap and an increased number of inflammatory cells. The stable plaque, on the other hand, is characterised by a smaller amount of lipids, more collagen and calcium and less inflammatory cells. Apart from being able to quantify the size of the plaque, a useful imaging modality should also be able to analyse plaque composition.

The plaque can be characterised in two different dimensions, size and echogenicity by using externally applied ultrasound on the carotid arteries. Both size [1] and echogenicity [1–4] of carotid plaques have been shown to predict not only stroke, but also other clinical manifestations of atherosclerosis, such as acute coronary syndromes including myocardial infarction. Thus, both of these plaque

dimensions are of interest for risk stratification. Analyses of carotid plaque specimens after endarterectomy indicates that the echogenicity reflects the different histopathological components, with echolucent plaques being associated with a high lipid and haemorrhage content [5].

While several studies have found that traditional risk factors, such as age, male gender, cigarette smoking, systolic blood pressure, high serum cholesterol, diabetes mellitus and low levels of HDL [6–10] are associated with carotid plaque size, determinants of plaque echogenicity are less well established. The presence of echolucent carotid plaques has been associated with low HDL levels [11], and with elevated levels of fasting and postprandial triglyceride rich lipoprotein [3]. Echolucent plaques in the femoral artery have also been found to be related to high levels of oxidised LDL and CRP [12]. Thus, lipid oxidation and inflammation may be important risk factors for plaque echogenicity.

Our hypothesis was that plaque size and echogenicity are associated with different risk factors. Earlier studies indicate that plaque echolucency is associated with lipid status [11]. Since both inflammation and oxidative stress have been advocated to play an important role in the development of atherosclerosis [13], several markers of inflammation and oxidative stress were investigated along with traditional risk factors. Treatment with statins are believed to induce regression of established atherosclerotic lesions, lower CRP levels [14], and possibly affect plaque composition [15]. Therefore, adjustment for statin treatment was made in all analyses.

Our aim was to investigate the relationship between carotid artery plaque size and echogenicity and cardiovascular risk factors, including markers of inflammation and oxidation in the PIVUS study, a population based cohort. We used the Prospective Investigation of the Vasculature in Uppsala Seniors (PIVUS) study, initiated in more than 1,000 subjects aged 70 living in the community of Uppsala, Sweden [16], to study the relationships.

Materials and Methods

For a detailed description of the basic characteristics of the PIVUS cohort see the previous publication by Stenborg et al. [17].

Subjects

The Prospective Investigation of the Vasculature in Uppsala Seniors (PIVUS) study is a population based cohort. All 70 year olds living in the community of Uppsala, Sweden were eligible. They received an invitation letter within 2 months of their 70th birthday. Of the total of 2,025 invited, 1,016 subjects participated giving a participation rate of 50, 1%. The study was approved by the ethics committee of the University of Uppsala and the participants gave informed consent. The present study was based on the 942 individuals in whom ultrasound pictures of high quality were obtained.

Basic Investigation

The participants answered a questionnaire disclosing their medical history, smoking habits and medication. All subjects were investigated in the morning after an over-night fast. Neither medication nor smoking was allowed after midnight preceding the investigations. Recordings of height, weight, abdominal and hip circumference were performed. Blood pressure was measured using a calibrated mercury sphygmomanometer in the non-cannulated arm after at least 30 min of rest in the supine position. The average of three recordings was used. Lipid variables and fasting blood glucose were measured by standard laboratory techniques. The Framingham risk score was calculated from these data [18].

Ultrasound System

The imaging was performed using an external B-mode ultrasound system (Acuson XP128 with a 10-MHz linear transducer, Acuson Mountain View, California, USA). All subjects were examined by the same ultrasonographer.

Ultrasound Procedure

The images were stored using the cine function during the electrocardiographic R-wave in the cardiac cycle. Frozen images of the arterial wall were saved and measurements subsequently performed on the stored digital images. Analyses were made using the AMS program package [19]. The common carotid artery, carotid bifurcation and internal carotid artery were examined bilaterally for the presence of plaques.

A local thickening of the intima-media by more than 50% compared with the surrounding IMT (but plaque area <10 mm^2) was defined as stage 1 plaques. Plaque volume exceeding 10 mm^2 was defined as stage 2, and presence of an increased velocity distal to the plaque measured with Doppler, indicating >50% stenosis, as stage 3.

A region of interest (ROI) was manually positioned around the largest plaque. The grey scale median (GSM) was calculated from pixel analysis within in the range 0–256. The blood was used as the reference for black and the adventitia as the reference for white. Plaques were defined as echolucent if GSM value was lower than 70, and as echorich if GSM was higher than 70.

Also, the area of the ROI was calculated. This analysis was carried out by the same ultrasonographer blinded to the clinical data.

Measurements of GSM and plaque size were repeated in 25 random subjects yielding coefficients of variation (CV) of 8.3 and 11.2%, respectively.

Insulin Resistance

Serum insulin was measured with an enzymatic-immunological assay (Boehringer Mannheim). HOMA insulin resistance index was defined as fasting blood glucose time's serum insulin/22.5.

Markers of Inflammation

High sensitive CRP was measured in human serum by an ultra sensitive particle enhanced immunoturbidimetric assay (Orion Diagnostica, Espoo, Finland) on a Konelab 20 autoanalyser (Thermo Clinical Labsystems, Espoo, Finland). The inter assay CV was 3.2%.

Cytokines, chemokines and adhesion molecules were analysed on the Evidence® array biochip analyser (Randox Laboratories, Ltd., Crumlin, UK).

The functional sensitivity for the different inflammatory markers was as follows: interleukin 6 (IL-6): 0.3, interferon-gamma (InfG): 1.8, tumour necrosis factor alpha (TNF-alpha): 1.8, monocyte chemotactic protein 1 (MCP-1): 19.4, intercellular adhesion molecule 1 (ICAM-1): 18.6, vascular cell adhesion molecule 1 (VCAM-1): 3.1, endothelial selectin (e-selectin): 3.1, platelet-selectin (p-selectin) : 11.2, lymphocyte selectin (l-selectin): 32.8, C reactive protein (CRP): 0.1, leukocyte count: 0.2.

Markers of Oxidative Stress

Baseline conjugated dienes of LDL (BCD-LDL) were measured by using a previously validated method (see Ref. [20] for details). The within assay CV for BCD-LDL was 4.4% and the between assay precision was 4.5%. Enzyme-linked immunoabsorbent assay (ELISA) kits (Mercodia, AB) were used to determine serum oxidised LDL, within-assay variation coefficient was 6.3% and between-assay CV was 4.7%. All glutathione indices, conjugated dienes (CD) and total antioxidant capacity (TAOC) values were measured and calculated as previously described [20]. Homocysteine was measured by using Enzyme Immunoassay method (Axis-Shield Diagnostics Ltd) with an intra-assay CV of 6.8%.

Statistics

IL-6, TNF alfa, InfG, e-selectin and homeostasis model assessment (HOMA) index were log-transformed in order to achieve a normal distribution. ANOVA was used to evaluate differences between groups after adjustment for gender, with the Bonferroni post-hoc test using a pre-specified number of tests (abnormal groups versus the normal group). All analyses were adjusted for the influence of gender and statin use. Two-tailed significance values were given and $P < 0.05$ was regarded as significant. StatView (SAS Inc., NC, USA) was used for the statistical calculations.

Results

Approximately 6% of the cohort reported a history of myocardial infarction, 2% reported stroke and 9% reported diabetes mellitus. Almost half of the cohort reported some cardiovascular medication (45%), with antihypertensive medication being the most prevalent (31%).

Fifteen percent reported use of statins, while insulin and oral antiglycemic drugs were reported in 2 and 6% respectively (Table 1).

Evaluation of Non-Participants

As the participation rate in this cohort was only 50%, we carried out an evaluation of cardiovascular disorders and medications in 100 consecutive non-participants. The prevalence of cardiovascular drug intake, history of myocardial infarction, coronary revascularisation, antihypertensive

Table 1 Basic characteristics of the study sample (%)

Gender	Total $n = 942$	Men $n = 469$	Women $n = 473$
Hypertension[a]	678 (72%)	328 (70%)	350 (74%)
Statin use	141 (15%)	75 (16%)	66 (14%)
Heart failure	38 (4%)	19 (4%)	14 (3%)
Diabetes mellitus[b]	85 (9%)	52 (11%)	33 (7%)
Previous myocardial Infarction[c]	57 (6%)	84 (8%)	14 (3%)
Previous smoker	396 (42%)	230 (49%)	166 (35%)
Current smoker	94 (10%)	47 (10%)	52 (11%)
Use of Cardiovascular Medication	424 (45%)	211 (45%)	213 (45%)
Use of antihypertensive medication	292 (31%)	145 (31%)	147 (31%)
Previous stroke[d]	19 (2%)	14 (3%)	5 (1%)

[a] Defined as systolic blood pressure >140 or diastolic >90, or use of antihypertensive medication

[b] Defined as history of diabetes mellitus or fasting sugar level >6.1

[c] Previous myocardial infarction which required hospitalisation

[d] Previous stroke which required hospitalisation

medication, statin use and insulin treatment were similar to those in the investigated sample, while the prevalence of diabetes, congestive heart failure and stroke tended to be higher among the non-participants.

Prevalence of Carotid Artery Plaque

The prevalence of unilateral plaque, defined as at least 50% increase of the intima-media thickness, in the carotid arteries was 34%, while the prevalence of bilateral plaques was 27%. Plaques were in general slightly more prevalent in men ($n = 322$) than in women ($n = 293$).

Individuals with carotid artery plaques had significantly higher Framingham risk score ($P < 0.0001$), higher levels of systolic blood pressure ($P < 0.0001$), higher levels of fasting blood glucose ($P = 0.02$), LDL/HDL-ratio ($P = 0.04$), ApoB/A1-ratio ($P = 0.013$), LDL ($P = 0.02$) and also a higher average of pack-years of cigarette smoking ($P = 0.008$) after adjustment for gender and statin use. No significant differences were seen for HDL-cholesterol, serum triglyceride level, DBP or body mass index (Table 2).

Relationships Between Plaque Size, Traditional Risk Factors, Markers of Oxidative Stress and Inflammation

A similar picture emerged, as described above, when traditional risk factors were related to plaque size and not to plaque prevalence. Framingham risk score, SBP, blood glucose, ApoB/A1-ratio, HOMA resistance index and smoking were significantly related to plaque size after adjustment gender and statin use. However, these relationships were not all significant in the post-hoc analysis in the group with flow-restricting plaque since there were only 27 subjects in this group. No significant relationships

were seen between plaque size and LDL-cholesterol, HDL-cholesterol, LDL/HDL-ratio ($P = 0.07$), serum triglycerides, DBP, or body mass index (Table 3).

BCD LDL levels were significantly higher, among subjects with plaques. Conversely, the total antioxidant capacity (TAOC) was lower among these subjects as could be expected. There was a trend of decreased BCD levels with increased plaque size, however non-significant in the post hoc analysis. OxLDL levels showed a stepwise increase with increasing plaque size. No other markers of oxidative stress were significantly related to plaque size.

TNF alpha, l-selectin (inversely) and leukocyte count and all markers of inflammation, were significantly related to plaque size after adjustment for gender and statin use (Table 3).

Relationships Between Plaque Echogenicity and Traditional Risk Factors and Markers of Oxidative Stress and Inflammation

Echolucent plaques were correlated with an increased Framingham risk score, elevated systolic blood pressure and higher BMI after adjustment for gender and statin use, while the levels of HDL were lower. Previous or present smoking was common in both groups of echolucent and echorich plaques with significantly more pack-years related to the echorich plaques. None of the other traditionally risk factors were significantly related to plaque echogenecity (Table 4).

Levels of total glutathione (TGSH), reduced glutathione (GSH) and oxidised glutathione (GSSG) were significantly lower among the subjects with echolucent plaques when compared to individuals with echorich plaques. None of the other markers of inflammation and oxidative stress were significantly related (Table 4).

Discussion

The main result of this study was that different risk factors were related to the two different dimensions of carotid plaque, i.e. size and echogenicity. HDL-cholesterol, body mass index and glutathione were related to echogenicity; while markers of inflammation were related to plaque size and not to echogenicity.

Relationships Between Plaque Occurrence and Size and Cardiovascular Risk Factors

Plaque occurrence and size was associated with systolic blood pressure, fasting blood glucose, LDL, LDL/HDL-ratio, ApoB/A1-ratio and smoking in this population. These are established risk factors for carotid atherosclerosis.

Table 2 Traditional risk factors in subjects with and without carotid artery plaques

	No plaque	Plaque	P-value
No.	327	594	
Framingham	10.4 ± 3.2	11.5 ± 3.4	<0.0001
SBP (mmHg)	146 ± 22	152 ± 23	<0.0001
LDL-cholesterol (mmol/l)	3.3 ± 0.9	3.4 ± 0.9	0.02
LDL/HDL-ratio	2.3 ± 0.8	2.4 ± 0.8	0.04
ApoB/A1-ratio	0.64 ± 0.2	0.67 ± 0.1	0.013
Fasting blood glucose (mmol/l)	5.1 ± 1.1	5.4 ± 1.7	0.02
Cigarette smoking (package years)	10 ± 15	13 ± 17	0.008

Differences between groups were calculated with ANOVA, adjusted for gender and statin use. Values presented as means ± SD

SBP systolic blood pressure, *DBP* diastolic blood pressure, *LDL* low density lipoprotein, *ApoA1* apolipoprotein A1, *ApoB* apolipoprotein B

Table 3 Relationships between plaque size and traditional risk factor and markers of inflammation and oxidation

	No plaque	Plaque <10 mm^2	Plaque >10 mm^2	Flow-obstructing plaque	P-value
No.	327	147	420	27	
Framingham risk score	10.4 ± 3.2	10.4 ± 3.2	11.8 ± 3.4***	12.7 ± 2.5**	<0.0001
SBP (mmHg)	145 ± 22	146 ± 23	153 ± 22***	157 ± 25*	<0.0001
Blood glucose (mmol/l)	5.2 ± 1.2	5.2 ± 1.1	5.5 ± 1.9*	5.6 ± 1.9	0.04
Cigarette smoking (pack years)	10.1 ± 15	12.7 ± 16	13 ± 17.5	20.5 ± 21*	0.0042
ApoB/ApoA1-ratio	0.64 ± 0.2	0.64 ± 0.2	0.67 ± 0.2*	0.70 ± 0.2	0.02
OxLDL (U/l)	129 ± 50	126 ± 45	137 ± 46	153 ± 56	0.002
TNFa (pg/ml)	1.4 (2.4–7.7)	1.4 (2.3–6.7)	1.4 (2.3–7.4)	1.7 (2.5–14.9)	0.03
l-Selectin (ng/l)	742 ± 145	707 ± 129	712 ± 141*	709 ± 149	0.038
BCD-LDL (μmol/l)	20.8 ± 7.2	23 ± 7.6*	21.5 ± 7.2	22.4 ± 7.8	0.011
TAOC (%)	38.1 ± 4	36.9 ± 4*	37.5 ± 4	38.2 ± 3	0.02
GSSG (μg/ml)	76 ± 38	83 ± 42	74 ± 33	68 ± 17	0.05
Leucocyte count	5.5 ± 1.3	5.5 ± 1.4	5.7 ± 1.5	6.5 ± 1.8*	0.005
HOMA resistance index (mmol/l* mU/l)	1.58 (0.90–3.44)	1.67 (0.8–4.1)	1.76 (0.87–4.4)	1.95 (1.1–9.2)	0.04

Data are given as means ± SD, or as median with 10–90th percentile in parenthesis. Differences between groups were calculated with ANOVA with adjustment for gender and statin use

* $P < 0.01$; ** $P < 0.001$; *** $P < 0.0001$ refer to post hoc analyses versus group with no plaque

SBP systolic blood pressure, *DBP* diastolic blood pressure, *LDL* low density lipoprotein, *HDL* high density lipoprotein, *ApoA1* apolipoprotein A1, *ApoB* apolipoprotein B, *BMI* body mass index, *OxLDL* oxidised low density lipoprotein, *BCD LDL* baseline conjugated diens of low density lipoprotein, *TAOC* total antioxidant capacity, *CD* conjugated diens, *TGSH* total glutathione, *GSH* reduced glutathione, *GSSG* oxidised glutathione, *IL-6* interleukin 6, *TNF alfa* tumour necrosis factor alfa, *IntfG* interferon gamma, *VCAM-1* vascular cell adhesion molecule 1, *ICAM-1* intercellular adhesion molecule, *MCP-1* monocyte chemotactic protein 1, *CRP* C-reactive protein, *HOMA index* homeostasis model assessment index

Table 4 Relationships between echolucent and echorich plaques and traditional risk factors, markers of inflammation and oxidative stress

	Echolucent plaque n = 304	Echorich plaque n = 290	P value
Framingham risk score	11.8 ± 3.3	11.2 ± 3.4	0.04
SBP	153.4 ± 23.2	150.4 ± 22.7	0.005
HDL	1.44 ± 0.4	1.56 ± 0.5	0.047
Smoking, pack years	12.6 ± 17.4	15.7 ± 17.5	0.005
BMI	27.1 ± 4.0	26.5 ± 4.3	0.05
TGSH	887 ± 178	920 ± 222	0.03
GSH	816 ± 169	840 ± 212	0.0002
GSSG	71 ± 29	82 ± 40	0.0001

Data are given as mean value ± SD and *P*-value. Differences between groups were calculated with ANOVA with adjustment for gender and statin use. Echolucent plaques are defined as plaques with a GSM value of less than 70, and echorich plaques as plaques with a GSM value of more than 70

SBP systolic blood pressure, *HDL* high density lipoprotein, *BMI* body mass index, *TGSH* total glutathione, *GSH* reduced glutathione, *GSSG* oxidised glutathione

These findings being consistent with several earlier studies of asymptomatic carotid arteriosclerosis [7, 8, 21, 22].

In the present study, plaque size was also related to increased levels of TNF alpha, a high leukocyte count and low levels of l-selectin. The relationship between with carotid atherosclerosis and inflammatory processes has previously been investigated. In the Rotterdam Study, increased fibrinogen was an independent predictor of carotid artery stenosis [7]. An elevated leukocyte count was associated with carotid arteriosclerosis in a study by Elkind and co-workers [23]. While high leukocyte count has been shown to predict future cardiovascular events [24, 25], less is known of the effects of l-selectin on atherosclerosis. However, in a small study, l-selectin levels were shown to be reduced in patients with ischemic heart disease compared to controls, and it was hypothesised that the chronic inflammatory process of atherosclerosis leads to downregulation of leucocyte expression of l-selectin, thus rendering lower circulating l-selectin levels. This hypothesis is supported by in vitro studies demonstrating that stimulation of leucocytes leads to a rapid downregulation of surface l-selectin expression [26].

The oxidative stress markers TAOC was reduced and BCD-LDL was increased in subjects with small plaques but not in those with larger plaques. This may be a pattern associated with the early stages of plaque growth, but it remains to be confirmed.

OxLDL levels have previously been associated with carotid artery atherosclerotic plaques [27], and we could confirm that carotid plaque size was linked to OxLDL in a stepwise fashion in the present study.

It should be emphasised that the vast majority of plaques observed in the present study are quite small and do not cause any obstruction of flow. Therefore, the present study should be regarded as a study of asymptomatic atherosclerosis. Other results could have been obtained if subjects with clinically important or symptomatic carotid stenosis had been investigated.

Relationships Between Plaque Echogenicity and Cardiovascular Risk Factors

The ANOVA analysis demonstrated that some of the risk factors related to plaque size were also related to the plaque echogenicity, while other risk factors were related to echogenicity alone. Body mass index, HDL and glutathione metabolism were factors mainly related to plaque echogenicity. Low levels of HDL-cholesterol have previously been shown to be associated with echolucent plaques, as well as with plaque progression [11]. In the present study, HDL was associated with the echogenicity but not size. HDL is known to be as a reverse cholesterol transporter and by the removal of cholesterol from the plaque and also other actions of HDL, such as anti-inflammatory actions, may influence the echogenicity of plaques.

Glutathione, an intracellular antioxidant widely distributed in human tissues, acts as a cofactor for glutathione peroxidase. This enzyme reduces H_2O_2, a reactive oxidant spices produced during the course of metabolism, to oxidised glutathione (GSSG) and water [28]. The GSSG level could be a result of a low glutathione peroxidase activity, indicating a reduced antioxidant activity in turn resulting in more echolucent plaques.

Another interesting finding was that BMI was associated with plaque echogenicity but not to plaque size. It has been shown that plaques that increase in GSM over time have a slow progress with respect to growth [29]. This finding could indicate that a good oxidative defence and a lean statue are characteristics that make a small plaque to increase in collagen and/or calcium content and in turn slowing down in plaque growth.

It has previously been reported that diabetics [30] and subjects with elevated inflammatory markers [31] have an increased prevalence of echolucent plaques. We could however not confirm these findings. Conversely, blood glucose levels and insulin resistance was related to plaque size, and none of the large number of inflammatory markers measured in this study were related to GSM in the plaque.

Limitation of the Study

The present sample is limited to Caucasians aged 70. Caution should therefore be made to draw conclusions to other ethnic and age groups.

The present study had a moderate participation rate. Therefore, we carried out an evaluation of cardiovascular disorders and medications in 100 consecutive subjects who refused to participate [13]. The prevalence of cardiovascular drug intake, history of myocardial infarction, coronary revascularisation, antihypertensive medication, statin use and insulin treatment were similar to those in the investigated sample, while the prevalence of diabetes, congestive heart failure and stroke tended to be higher among the non-participants.

A large number of variables were evaluated in order to obtain a broad view on risk factors for plaque size and echogenicity. This approach does however increase the risk of false positive findings. Thus, the results need to be repeated in other cohorts.

In conclusion, of the two carotid plaque dimensions, size and echogenicity, Framingham risk score, systolic blood pressure, fasting blood glucose, HOMA insulin resistance, cigarette smoking, ApoB/A, OxLDL, TNF alpha, low levels of l-selectin, decreased TAOC, increased leucocyte count were related to size, while HDL-cholesterol, glutathione and body mass index mainly were related to echogenicity. These results suggest that plaque size and echogenicity partly are influenced by different risk factors.

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