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Evidence for Altered Inflammatory and Repair Responses in Symptomatic Carotid Plaques from Elderly Patients

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Abstract

Objective: Most acute cardiovascular events are caused by rupture of an atherosclerotic plaque. The incidence of cardiovascular events increases with age and inflammation is generally considered to be the main cause of increased plaque vulnerability. However, the relationship between age and plaque inflammation has not yet been fully clarified. The aim of our study was to determine if age-dependent plaque vulnerability is associated with increased plaque inflammation.

Methods: We collected 200 endarterectomy specimens, 103 of which were from patients 70 years or older. One-hundred and five patients had had a recent cerebrovascular event, whereas the rest were asymptomatic despite significant carotid stenosis. Smooth muscle cell, lipid and macrophage content were analyzed by histology. Cytokines, growth factors and extracellular matrix proteins were analyzed in whole plaque homogenates by immunoassays and biochemical methods.

Results: Plaques from elderly patients contained less IFN-γ, TNF-α, fractalkine, sCD40L, and elastin. Lipid and macrophage content was higher in plaques from symptomatic compared to asymptomatic patients in the elderly group, but not in younger patients. The elastin and collagen content was lower in plaques from symptomatic patients in both age groups. Plaques associated with symptoms also contained more TNF-α, IL-1β, IL-6, sCD40L, MIP-1β, MCP-1, RANTES and VEGF, regardless of age.

Conclusions: Our data imply that increased plaque vulnerability in the symptomatic elderly patients is associated with increased lipid accumulation and impaired tissue repair, rather than with increased plaque inflammation, compared to younger individuals.

1. Introduction

Rupture or erosion of carotid atherosclerotic plaques with subsequent thrombosis, embolization and cerebral blood flow obstruction is one of the main mechanisms behind ischemic stroke or transitory ischemic attacks (TIA). The current standard of care for these patients is surgical removal of ipsilateral atherosclerotic plaques occluding more than 50-70% of the vascular lumen, in addition to platelet inhibition and lipid lowering strategies.[1] Endarterectomy is also performed on carotid plaques with a stenosis grade of more than 80% in asymptomatic patients, as it has been shown to reduce the subsequent risk of stroke.[2] However, high co-morbidity and perioperative risk restrict the indications of surgery in elderly patients, meaning that a larger number of individuals in this patient group are assigned to conservative medical treatment.

Defining the mechanisms involved in plaque vulnerability is central for designing efficient treatment protocols for prevention of ischemic cerebrovascular events. Atherosclerotic plaques characterized by high levels of inflammatory activity are considered to be vulnerable and prone to rupture.[3] Carotid plaques collected from elderly patients have a more vulnerable histological phenotype characterized by larger lipid cores, lower smooth muscle cell and fibrous tissue content.[4-6] However, the extent of inflammatory burden of atherosclerotic plaques in elderly compared to younger patients is still controversial. Whereas Redgrave *et al.* recorded a tendency towards decreased carotid plaque macrophage and lymphocyte infiltration with advancing age,[4] two other studies did not find any significant relationship between macrophage content and age.[5, 6] Moreover, the immunohistochemical evaluation of macrophage and lymphocyte infiltration in carotid plaques does not offer information on the state of activation of these cells, adding another level of uncertainty to this matter.

The purpose of our study was to perform an in-depth comparison of inflammatory status in carotid plaques collected from young versus elderly patients, in relationship with symptoms and histological characteristics of plaque vulnerability. Previous studies mostly used histological techniques. By using biochemical analysis of whole plaque homogenates we measured the amounts of inflammatory cytokines, chemokines and growth factors in 200 carotid endarterectomy specimens. We compared plaque composition between young (<70 years of age) versus elderly (≥ 70 years of age) and symptomatic versus asymptomatic patients.

2. Methods

For a more detailed description of the methods, please see the Supplemental methods section.

2.1 Study population

Two hundred plaques were collected from 197 patients undergoing carotid endarterectomy. Indications for surgery were plaques associated with ipsilateral symptoms (TIA, stroke or amaurosis fugax) and carotid stenosis greater than 70%, or plaques causing carotid stenosis of > 80% in the absence of symptoms. Patients with ipsilateral carotid artery occlusion or restenosis after previous carotid endarterectomy were excluded. Symptomatic patients who had a delay of more than one month between symptoms and surgery were also excluded, to avoid symptomatic plaques having time to stabilize before analysis.[7] The presence of hypertension (systolic blood pressure >140 mm Hg), diabetes, smoking and the use of statins, beta blockers, angiotensin converting enzyme inhibitors (ACEI), angiotensin II receptor blockers (ARB), acetylsalicylic acid (ASA), and other platelet inhibitors (clopidogrel and dipyridamole) were recorded. A blood sample was collected from each patient the day before endarterectomy. The study was approved by the local ethical committee and informed

consent was obtained from each patient. The study participants were divided into young and elderly using as cut-off the median age (70 years).

2.2 Preparation of plaques

Plaques were snap-frozen in liquid nitrogen directly after removal. A 1-mm-thick segment from the most stenotic region of the plaque was removed for histology and immunohistochemistry. The rest of the plaque was weighed and homogenized as described earlier.[8]

2.3 Histology and immunohistochemistry

Plaque sections (8 μm thick) were stained for lipids (Oil Red O), macrophages (CD68) and smooth muscle cells (α-actin) as previously described.[9]

2.4 Cytokine, chemokine and growth factor analysis

The following cytokines, chemokines and growth factors were analyzed according to the manufacturer's instructions (Human cytokine/chemokine immunoassay, Millipore Corporation, MA) in aliquots of plaque homogenate: interleukin 6 (IL-6), interleukin-12 (p70) (IL-12(p70)), interleukin-12(p40) (IL-12(p40)), macrophage inflammatory protein-1β (MIP-1β), platelet-derived growth factor AB/BB (PDGF-AB/BB), regulated on activation normal T-cell expressed and secreted (RANTES), vascular endothelial growth factor (VEGF), interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), monocyte chemoattractant protein-1 (MCP-1), interleukin-1β (IL-1β), fractalkine, eotaxin and soluble CD40 ligand (sCD40L).

2.5 Collagen and elastin assessment in plaque homogenate

Elastin was measured using the Fastin Elastin assay (Biocolor, Carrickfergus, Northern Ireland, UK) and collagen (acid-soluble and pepsin-soluble collagens types I to V) was measured using Sircol soluble Collagen assay (Biocolor, Carrickfergus, Northern Ireland, UK) in plaque homogenates, according to the manufacturer's instructions. All components measured in plaque homogenate were normalized to plaque wet weight (pg or mg/g).

2.6 Statistics

Skewed variables were logarithmically transformed. Differences in baseline characteristics in relationship with age and symptoms were assessed by 2-way ANOVA. The differences in plaque components in relationship with age and symptoms were assessed by using unadjusted 2-way ANOVA (Model A) and 2-way ANCOVA adjusted for conventional cardiovascular (CV) risk factors, creatinine, and medication (Model B) or for CV risk factors, creatinine, medication, white blood cell count (WBC), and high-sensitivity CRP (hsCRP) (Model C). A significant interaction between age and symptoms for a particular plaque component indicates that the difference between symptomatic and asymptomatic plaques with regard to that component is only significant within a certain age group. A *P*-value of less than 0.05 was considered significant. IBM SPSS 21.0 software was used for all statistical analyses.

3. Results

3.1 Population characteristics

The youngest patient included in the study was 41 years old and the oldest 88, with a median age of 70. Subjects 70 years of age or older were defined as "elderly" (n=103) and the rest as "younger" (n=96). Each age group was further divided into symptomatic and asymptomatic patients. There were more symptomatic patients in the elderly compared to the younger group

(Table 1). The mean age of the symptomatic patients was significantly higher than the mean age of the asymptomatic patients within the elderly group, whereas there was no difference between subgroups within the younger population (Table 1). A significantly higher percentage of the symptomatic patients had diabetes mellitus compared to asymptomatics across the age groups. The elderly subjects had lower circulating WBC and higher creatinine levels compared to the younger group. There were significantly fewer smokers in the elderly group compared to the younger, but there was no association between smoking status and the prevalence of symptoms in the population. There were no significant differences among the groups with regard to body mass index (BMI), hypertension, low-density lipoprotein (LDL), high-density lipoprotein (HDL) or use of statins, beta blockers, ACEI, ARB, ASA or other platelet inhibitors (Table 1).

3.2 Plaque components

Plaques associated with symptoms in the elderly group were characterized by higher lipid and macrophage content compared to plaques from asymptomatic patients (Table 2, Model A). In contrast, no differences in these components could be observed in the younger group. The matrix proteins elastin and collagen were lower in plaques related to symptoms in both age groups. Plaques collected from the elderly patients contained significantly less elastin but similar amounts of collagen compared with the younger group (Table 2, Model A). The differences in plaque macrophages and lipids between symptomatic and asymptomatic elderly patients and the difference in plaque elastin between elderly and younger patients was independent of CV risk factors, kidney function and medication (Table 2, Model B) and remained significant after additional adjustment for the markers of systemic inflammation WBC and hsCRP (Table 2, Model C). Similarly, the difference in elastin between plaques

7

from symptomatic and asymptomatic patients remained significant in the fully adjusted model (Table 2, Model C).

3.3 Plaque cytokines, chemokines and growth factors

Plaques collected from elderly patients contained lower amounts of IFN-γ, TNF-α, fractalkine, and sCD40L (Table 3, Model A). The age-related difference in sCD40L remained significant after correction for traditional CV risk factors, creatinine and medication (Table 3, Model B) but lost significance after additional adjustment for WBC and CRP (Table 3, Model C). The differences in plaque IFN-γ, TNF-α and fractalkine between young and elderly were independent of all the potential covariates considered.

Plaques associated with symptoms were richer in TNF-α, IL-6, sCD40L, MIP-1β, MCP-1, RANTES, and VEGF in both age groups (Table 3, Model A). The relationships between symptoms and plaque IL-6, TNF-α, MIP-1β, MCP-1 and RANTES were independent of CV risk factors, plasma creatinine, medication, WBC and CRP (Table 3, Model C). However, the differences in sCD40L and VEGF between plaques from symptomatic and asymptomatic patients lost significance after adjustment (Table 3, Model B), suggesting that these relationships are at least partially explained by variations in CV risk profile, kidney function or medication. Except for VEGF, the observed differences in plaque composition between symptomatics and asymptomatics were irrespective of age, as there was no significant interaction between age and symptoms in the analysis.

4. Discussion

The purpose of our study was to assess the role of inflammation in atherosclerotic plaque vulnerability at advanced age. We measured a large number of cytokines, chemokines, growth

factors and matrix proteins in whole human carotid plaque homogenates. This novel approach allowed us to evaluate the concentration of each compound in the entire plaque, rather than evaluating a limited number of plaque sections by immunohistochemistry. We found that carotid plaques in the elderly contain lower amounts of pro-inflammatory cytokines and of the matrix protein elastin compared to plaques from younger patients. In contrast, plaque lipid and macrophage content were highest in plaques from symptomatic elderly. A large lipid pool with high cholesterol saturation may contribute to plaque rupture due to cholesterol crystallization[10], leading to volume expansion and mechanical damage of the fibrous cap.[11] Additionally, we found that plaques associated with symptoms were generally more inflammatory and contained less collagen and elastin, regardless of age.

With age, complex changes in the immune system take place, termed immunosenescence. The ability to fight infections and to mount adequate immune responses to vaccination declines,[12] there is a contraction of the naïve T cell repertoire and a dominance of Th2 over Th1 responses.[13] In our material, carotid plaques collected from the elderly patient group contained lower amounts of IFN-γ, TNF-α, fractalkine, and sCD40L compared to plaques of younger patients. IFN-γ is a marker of Th1 cell activation[14] and TNF-α is the signature cytokine of activated macrophages.[15] Fractalkine is released by endothelial cells activated by TNF-α and IL-1β and is a potent chemoattractant for T cells and monocytes.[16] sCD40L is a pro-inflammatory molecule involved in atherothrombosis.[17] These data suggest that elderly plaques are less inflammatory, which may be related to the above-mentioned patterns of immunosenescence. In support of this, peripheral blood mononuclear cells from postmenopausal women were shown to proliferate slower and produce significantly less INF-γ upon stimulation when compared to cells from young or middle aged women.[18] Agius *et al.* have found that decreased macrophage TNF-α secretion in elderly individuals prevents CD4+

T-cell entry into the skin upon antigen stimulation, leading to impaired immunosurveillance.[19] The reduced concentration of TNF-α in elderly plaques in our study contrasts to the increased macrophage content, suggesting that the infiltrating macrophages in elderly are less active, and further emphasizing the importance of using quantitative biochemical techniques rather than only immunohistochemical methods, sometimes semi-quantitative, for assessing differences in plaque composition.

Plaques associated with symptoms in both groups contained lower levels of elastin and collagen. There was also a trend towards lower SMC staining in these plaques independently of age, in line with previously published data.[20, 21] We found higher levels of the monocyte chemoattractants MCP-1, MIP-1β and RANTES and of the monocyte/macrophage-related cytokines TNF-α and IL-1β in plaques from symptomatic patients, regardless of age. Additionally, these lesions contained higher amounts of the pro-inflammatory and pro-thrombotic molecule sCD40L[17] and more VEGF, which is a stimulus for plaque neovascularization.[22] Th1 cells and their cytokines are considered to be pro-inflammatory and pro-atherogenic and contribute to plaque vulnerability and rupture.[23] However, we did not find any relationship between the signature cytokines of Th1 lymphocytes, IFN-γ and IL-12, and symptoms. This finding is in line with our previously published study suggesting that a lower systemic Th2 lymphocyte activity, rather than an increased Th1 activation, is related to increased risk for CV events.[24] Nevertheless, there might be important differences between the systemic and the local immune environment, and the Th2-specific cytokines IL-4 and IL-5 were not measured in this material.

There have been earlier attempts to clarify the characteristics of plaque composition responsible for the increasing incidence of cerebrovascular events with advancing age. There

is currently an established consensus regarding the histological features of carotid atherosclerosis in the elderly, with several authors demonstrating a higher prevalence of plaques with large lipid cores, lower prevalence of fibrous plaques, decreased smooth muscle cell content and increased plaque calcification in this population.[4-6] In the present work we have used quantitative biochemical measurements of extracellular matrix proteins in whole plaque homogenates, which allowed us to accurately evaluate plaque content of collagen and elastin separately. Plaque collagen content did not vary significantly with increasing age, which is in line with previously published data by others.[5, 6] In contrast, we detected a significant decrease of the elastin content in carotid plaques from elderly compared with younger patients, which confirms previous findings by our group.[8] These data suggest that the different extracellular matrix proteins reflect different processes related to plaque stability and hint towards defective repair mechanisms, mainly affecting elastin turnover, in the elderly.

The inflammatory activity of carotid plaques in young and elderly was previously assessed by immunohistochemical staining of intraplaque macrophages and lymphocytes, with conflicting results. Van Lammeren *et al.* have detected a tendency towards increasing macrophage infiltration with increasing age in a study cohort consisting of approximately fifteen percent asymptomatic and eighty-five percent symptomatic patients.[5] In contrast, Redgrave *et al.* showed that carotid plaques having advanced inflammation were less prevalent with increasing age, in a study focused exclusively on symptomatic patients.[4] Our more evenly balanced study population allowed us to study plaque composition both with regard to symptoms and age group. We found macrophage content to be significantly increased in plaques associated with symptoms, mainly due to high levels of macrophage infiltration in the elderly symptomatic sub-population. However, counting infiltrating cells alone offers little information about their state of activation. An early study, focused on the role of

inflammatory cells in plaque vulnerability, suggested that the state of activation of infiltrating macrophages and lymphocytes, assessed by HLA-DR staining, is associated with carotid plaque rupture.[25] Our study adds important information regarding the concentrations of a large number of inflammatory cytokines and chemokines in atherosclerotic carotid plaques in elderly compared to younger patients undergoing carotid endarterectomy. Cytokine and chemokine levels are downstream results of the complex interactions between different cell populations infiltrating the plaques and accurately reflect the inflammatory state of the entire plaque. Our results are in line with the current consensus stating that vulnerable plaques are more inflammatory. Additionally, we demonstrate that this effect is valid throughout the age spectrum. Plaques collected from elderly patients contained lower levels of several inflammatory cytokines, as a group, suggesting that the increased plaque vulnerability with increasing age is due to other mechanisms, rather than to increased local inflammatory activity.

5. Study limitations

The main limitation of our study is related to patient selection. Patients that were considered to have too high surgical risk were not operated, leading to a possible selection bias towards healthier elderly. Additionally, the analysis of ipsilateral carotid plaque specimens collected after an acute cerebrovascular event should be interpreted with due caution, as it is difficult to assess to which extent plaque rupture and the subsequent thrombosis have altered plaque composition. A high number of patients in the material were treated with statins, which may have attenuated plaque inflammation. However statin use was similar among the four groups, and most of the differences between groups remained statistically significant after additional adjustment for the use of statins and other medications. Our ex-vivo study is not mechanistic, which precludes the answer whether the lower amounts of cytokines and chemokines found in

the elderly plaques are related to systemic immunosenescence, lower degree of activation of the locally infiltrating leukocytes, or to other factors. The detailed mechanisms should be further evaluated in studies designed for that purpose.

6. Conclusions

In the present study we performed for the first time a thorough assessment of inflammatory status and plaque composition in whole carotid plaques of elderly patients, in comparison with a younger population and taking the presence or absence of symptoms into account. We demonstrate reduced inflammatory activity and elastin content in plaques of elderly individuals. Moreover, plaques from elderly symptomatic patients contained the highest amounts of lipids and macrophages among the studied carotid specimens. Plaques associated with symptoms were generally more inflammatory and contained lower levels of matrix proteins, regardless of age. These findings imply that plaque vulnerability leading to cerebrovascular events in the elderly is related to impaired mechanisms responsible for lipid clearance and tissue repair, rather than to increased local inflammation in the arterial wall.

Disclosures

None.

References

[1]		Barnett, HJ, Taylor, DW, Eliasziw, M, et al., Benefit of carotid endarterectomy in patients with symptomatic moderate or severe stenosis. North American Symptomatic Carotid Endarterectomy Trial Collaborators, N Engl J Med, 1998;339:1415-1425.
[2]		Halliday, A, Mansfield, A, Marro, J, et al., Prevention of disabling and fatal strokes by successful carotid endarterectomy in patients without recent neurological symptoms: randomised controlled trial, Lancet, 2004;363:1491-1502.
[3]		Naghavi, M, Libby, P, Falk, E, et al., From vulnerable plaque to vulnerable patient: a call for new definitions and risk assessment strategies: Part I, Circulation, 2003;108:1664-1672.
[4]		Redgrave, JN, Lovett, JK and Rothwell, PM, Histological features of symptomatic carotid plaques in relation to age and smoking: the oxford plaque study, Stroke, 2010;41:2288-2294.
[5]		van Lammeren, GW, Reichmann, BL, Moll, FL, et al., Atherosclerotic plaque vulnerability as an explanation for the increased risk of stroke in elderly undergoing carotid artery stenting, Stroke, 2011;42:2550-2555.
[6]		van Oostrom, O, Velema, E, Schoneveld, AH, et al., Age-related changes in plaque composition: a study in patients suffering from carotid artery stenosis, Cardiovasc Pathol, 2005;14:126-134.
[7]		Peeters, W, Hellings, WE, de Kleijn, DP, et al., Carotid atherosclerotic plaques stabilize after stroke: insights into the natural process of atherosclerotic plaque stabilization, Arterioscler Thromb Vasc Biol, 2009;29:128-133.
[8]		Goncalves, I, Moses, J, Dias, N, et al., Changes related to age and cerebrovascular symptoms in the extracellular matrix of human carotid plaques, Stroke, 2003;34:616-622.
[9]		Edsfeldt, A, Nitulescu, M, Grufman, H, et al., Soluble urokinase plasminogen activator receptor is associated with inflammation in the vulnerable human atherosclerotic plaque, Stroke, 2012;43:3305-3312.
[10]		Vedre, A, Pathak, DR, Crimp, M, et al., Physical factors that trigger cholesterol crystallization leading to plaque rupture, Atherosclerosis, 2009;203:89-96.
[11]		Abela, GS, Cholesterol crystals piercing the arterial plaque and intima trigger local and systemic inflammation, J Clin Lipidol, 2010;4:156-164.
[12]		Reber, AJ, Chirkova, T, Kim, JH, et al., Immunosenescence and Challenges of Vaccination against Influenza in the Aging Population, Aging Dis, 2012;3:68-90.
[13]		Desai, A, Grolleau-Julius, A and Yung, R, Leukocyte function in the aging immune system, J Leukoc Biol, 2010;87:1001-1009.
[14]		Schoenborn, JR and Wilson, CB, Regulation of interferon-gamma during innate and adaptive immune responses, Adv Immunol, 2007;96:41-101.
[15]		Parameswaran, N and Patial, S, Tumor necrosis factor-alpha signaling in macrophages, Crit Rev Eukaryot Gene Expr, 2010;20:87-103.
[16]		Liu, H and Jiang, D, Fractalkine/CX3CR1 and atherosclerosis, Clin Chim Acta, 2011;412:1180-1186.

[17]   Antoniades, C, Bakogiannis, C, Tousoulis, D, et al., The CD40/CD40 ligand system: linking inflammation with atherothrombosis, J Am Coll Cardiol, 2009;54:669-677.

[18]   Priyanka, HP, Sharma, U, Gopinath, S, et al., Menstrual cycle and reproductive aging alters immune reactivity, NGF expression, antioxidant enzyme activities, and intracellular signaling pathways in the peripheral blood mononuclear cells of healthy women, Brain Behav Immun, 2013;32:131-143.

[19]   Agius, E, Lacy, KE, Vukmanovic-Stejic, M, et al., Decreased TNF-alpha synthesis by macrophages restricts cutaneous immunosurveillance by memory CD4+ T cells during aging, J Exp Med, 2009;206:1929-1940.

[20]   Montecucco, F, Lenglet, S, Gayet-Ageron, A, et al., Systemic and intraplaque mediators of inflammation are increased in patients symptomatic for ischemic stroke, Stroke, 2010;41:1394-1404.

[21]   Verhoeven, B, Hellings, WE, Moll, FL, et al., Carotid atherosclerotic plaques in patients with transient ischemic attacks and stroke have unstable characteristics compared with plaques in asymptomatic and amaurosis fugax patients, J Vasc Surg, 2005;42:1075-1081.

[22]   Inoue, M, Itoh, H, Ueda, M, et al., Vascular endothelial growth factor (VEGF) expression in human coronary atherosclerotic lesions: possible pathophysiological significance of VEGF in progression of atherosclerosis, Circulation, 1998;98:2108-2116.

[23]   Halvorsen, B, Otterdal, K, Dahl, TB, et al., Atherosclerotic plaque stability--what determines the fate of a plaque?, Prog Cardiovasc Dis, 2008;51:183-194.

[24]   Engelbertsen, D, Andersson, L, Ljungcrantz, I, et al., T-helper 2 immunity is associated with reduced risk of myocardial infarction and stroke, Arterioscler Thromb Vasc Biol, 2013;33:637-644.

[25]   Carr, SC, Farb, A, Pearce, WH, et al., Activated inflammatory cells are associated with plaque rupture in carotid artery stenosis, Surgery, 1997;122:757-763; discussion 763-754.

Table 1. Clinical Characteristics of the Patients

	Younger (< 70 years of age)		Elderly (≥ 70 years of age)		2-way ANOVA		
	Asymptomatic	Symptomatic	Asymptomatic	Symptomatic	P^{age}	P^{sym}	P^{int}
Number of subjects (%)*	58 (60)	39 (40)	37 (36)	66 (64)			
Age (years)	63±6	62±6	73±3	77±4	<0.001	0.016	<0.001
Female gender, n (%)†	20 (34)	13 (33)	12 (32)	23 (35)	n.s.	n.s.	n.s.
Diabetes, n (%)†	13 (22)	19 (49)	9 (24)	25 (38)	n.s.	0.004	n.s.
BMI (kg/m^2)	26±4	27±4	27±4	26±4	n.s.	n.s.	n.s.
Hypertension, n (%)†	44 (76)	28 (72)	32 (86)	49 (74)	n.s.	n.s.	n.s.
Current smoker, n (%)†	26 (45)	21 (54)	13 (35)	7 (11)	<0.001	n.s.	0.010
Statin, n (%)†	55 (95)	33 (85)	32 (86)	55 (83)	n.s.	n.s.	n.s.
Beta blocker, n (%)†	32 (55)	18 (46)	18 (49)	30 (46)	n.s.	n.s.	n.s.

					P^{age}	P^{sym}	P^{int}
ACEI/ARB, n (%)†	34 (59)	22 (56)	21 (57)	25 (38)	n.s.	n.s.	n.s.
ASA, n (%)†	53 (91)	33 (85)	32 (86)	58 (88)	n.s.	n.s.	n.s.
Other platelet inhibitors, n (%)#	10 (17)	9 (23)	6 (16)	14 (21)	n.s.	n.s.	n.s.
LDL (mmol/L)	2.4±1.0	2.8±1.2	2.6±0.9	2.6±1.1	n.s.	n.s.	n.s.
HDL (mmol/L)	1.2±0.4	1.2±0.6	1.2±0.4	1.1±0.3	n.s.	n.s.	n.s.
TG (mmol/L)	1.6±0.8	1.6±0.7	1.4±0.8	1.3±0.7	0.045	n.s.	n.s.
Hb (g/L)	142±13	140±15	141±14	136±13	n.s.	n.s.	n.s.
WBC (x10^9/L)	8.3±1.8	8.4±2.4	7.3±1.9	7.5±1.8	0.001	n.s.	n.s.
Creatinine (µmol/L)	82±21	86±4	94±28	96±23	0.002	n.s.	n.s.
hsCRP (mg/L)	3.1 (1.5-5.0)	4.3 (2.4-6.1)	4.2 (2.3-6.9)	4.0 (1.9-9.3)	n.s.	n.s.	n.s.

Continuous variables are presented as mean ± standard deviation for normally distributed variables and median (interquartile range) for skewed variables.

BMI, body mass index; ACEI, angiotensin converting enzyme inhibitor; ARB, angiotensin II receptor blocker; ASA, acetylsalicylic acid; LDL, low density lipoprotein; HDL, high density lipoprotein; TG, triglycerides; Hb, hemoglobin; WBC, white blood cell count; hsCRP, high sensitive C-reactive protein. P^{age}, reflects differences between elderly and younger; P^{sym}, reflects differences between asymptomatic and symptomatic subjects; P^{int}, reflects interaction between

age and symptoms for the respective variable. * Percentage of the total younger and elderly patients, respectively. †Percentage of the total number of subjects

in the respective subgroup. # Clopidogrel or dipyridamole.

Table 2. Plaque Histology and Matrix Proteins

	Younger		Elderly		Non-adjusted Model A			Co-variate adjusted Model B			Co-variate adjusted Model C		
	Asympt	Sympt	Asympt	Sympt	P^{age}	P^{sym}	P^{int}	P^{age}	P^{sym}	P^{int}	P^{age}	P^{sym}	P^{int}
α-actin (% area)	25.5 (19.2-35.1)	21.5 (14.3-41.1)	25.9 (16.4-35.8)	18.8 (13.3-28.6)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Oil Red O (% area)	24.8±13.6	25.0±15.8	23.2±12.0	32.3±13.8	n.s.	0.023	0.031	n.s.	0.010	n.s.	n.s.	0.023	n.s.
CD68 (% area)	23.3±12.9	24.0±11.5	23.0±12.6	30.3±13.1	n.s.	0.030	n.s.	n.s.	0.008	n.s.	n.s.	0.010	n.s.
Elastin (mg/g)	59.2 (44.3-95.6)	54.0 (36.1-91.1)	54.4 (38.3-72.6)	40.7 (29.4-67.3)	0.031	0.037	n.s.	0.004	n.s.	n.s.	0.019	0.044	n.s.
Collagen (mg/g)	57.9 (31.0-79.6)	40.3 (28.6-53.9)	55.3 (38.5-69.7)	41.1 (29.0-65.0)	n.s.	0.055	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

Normally distributed variables are presented as mean ± standard deviation and skewed variables are presented as median (interquartile range). P^{age}, reflects differences between elderly and younger; P^{sym}, reflects differences between asymptomatic and symptomatic subjects; P^{int}, reflects interaction between age and symptoms for the respective variable. Model A – unadjusted. Model B - adjusted for gender, BMI, diabetes, hypertension, smoking, creatinine, triglycerides, LDL, HDL, and use of statins, beta blockers, ACEI, ARB, ASA or other platelet inhibitors. Model C – adjusted for the same covariates as Model B plus WBC and hsCRP.

Table 3. Plaque Cytokines, Chemokines, and Growth Factors (pg/g wet weight plaque)

	Younger		Elderly		Non-adjusted Model A			Co-variate adjusted Model B			Co-variate adjusted Model C		
	Asympt	Sympt	Asympt	Sympt	P^{age}	P^{sym}	P^{int}	P^{age}	P^{sym}	P^{int}	P^{age}	P^{sym}	P^{int}
TNF-α	207 (73-404)	439 (152-695)	106 (36-216)	224 (100-414)	<0.001	<0.001	n.s.	<0.001	0.004	n.s.	0.001	0.004	n.s.
IFN-γ	69 (20-135)	75 (35-182)	10 (0-113)	47 (9-114)	0.028	n.s.	n.s.	0.024	n.s.	n.s.	0.033	n.s.	n.s.
IL-12(p40)	0 (0-1.1)	0 (0-182.5)	0 (0-0)	0 (0-163.0)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s	n.s.
IL-12(p70)	33.4 (10. 9-75.4)	39.8 (0-80.1)	17.3 (0-45.9)	23.5 (0-48.4)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
IL-1β	0 (0-12.9)	0 (0-11.5)	0 (0-4.4)	0 (0-14.9)	n.s.	0.006	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

IL-6	700	1214	446	1431	n.s.	<0.001	n.s.	n.s.	0.002	n.s.	n.s.	0.002	n.s.
	(370-1594)	(605-2136)	(262-1307)	(746-2435)									
sCD40L	347	471	309	391	0.015	0.007	n.s.	0.047	n.s.	n.s.	n.s.	n.s.	n.s.
	(190-846)	(309-1205)	(94-608)	(190-1040)									
MCP-1	2035	4092	2099	4337	n.s.	n.s.	n.s.	n.s.	0.013	n.s.	n.s.	0.020	n.s.
	(1022-5225)	(1351-6055)	(858-8840)	(2530-8117)									
MIP-1β	177	230	127	362	n.s.	0.009	n.s.	n.s.	0.001	n.s.	n.s.	0.002	n.s.
	(26-306)	(63-430)	(0-325)	(157-641)									
RANTES	177	773	286	1030	n.s.	0.044	n.s.	n.s.	0.053	n.s.	n.s.	0.041	n.s.
	(0-1289)	(0-2260)	(0-1664)	(420-2188)									
Eotaxin	0	0	0	9.8	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.032
	(0-95.8)	(0-119.8)	(0-89.9)	(0-49.0)									
Fractalkine	1640	1746	1097	1057	0.005	n.s.	n.s.	0.010	n.s.	n.s.	0.030	n.s.	n.s.
	(738-3521)	(977-4052)	(420-2232)	(717-2204)									

PDGF-AB/BB	725 (352-1381)	819 (279-1184)	766 (407-1397)	792 (495-1282)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
VEGF	492 (94-1140)	854 (438-1390)	328 (0-1263)	551 (121-1449)	n.s.	0.020	n.s.	n.s.	n.s.	0.044	n.s.	n.s.	0.042

The amounts of plaque components were not normally distributed and are presented as median (interquartile range). P^{age}, reflects differences between elderly and younger; P^{sym}, reflects differences between asymptomatic and symptomatic subjects; P^{int}, reflects interaction between age and symptoms for the respective variable. Model A – unadjusted. Model B - adjusted for gender, BMI, diabetes, hypertension, smoking, creatinine, triglycerides, LDL, HDL, and use of statins, beta blockers, ACEI, ARB, ASA or other platelet inhibitors. Model C – adjusted for the same covariates as model B plus WBC and hsCRP.