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Circulating cytokines reflect the expression of pro-inflammatory cytokines in atherosclerotic plaques

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Abstract

Aims: Inflammation is a key factor in the development of plaque rupture and acute cardiovascular events. Although imaging techniques can be used to identify vulnerable atherosclerotic plaques, we are lacking non-invasive methods, such as plasma markers of plaque inflammation that could help to identify presence of vulnerable plaques. The aim of the present study was to investigate whether increased plasma levels of pro-inflammatory cytokines reflects inflammatory activity within atherosclerotic plaques.

Methods and Results: Cytokines were measured using Luminex immunoassay in 200 homogenized plaque extracts and plasma, obtained from 197 subjects undergoing carotid surgery. Plasma levels of macrophage inflammatory protein-1 β (MIP-1 β), tumor necrosis factor- α (TNF- α) and fractalkine correlated significantly, not only with plaque levels of the same cytokines but also with the abundance of several pro-inflammatory and atherogenic cytokines assessed in plaque tissue. High plasma levels (upper tertile) of MIP-1 β , TNF- α and fractalkine identified the presence of a plaque with high inflammation (above median of a score based on the plaque content of MIP-1 β , TNF- α , interferon- γ (IFN- γ) and fractalkine) with a sensitivity between 65 and 67% and a specificity between 78 and 83%. Furthermore, this study shows that high plasma levels of MIP-1 β , TNF- α and fractalkine predict future transient ischemic attacks.

Conclusions: Our findings show that the plasma levels of MIP-1 β , TNF- α and fractalkine reflect the levels of several pro-atherogenic cytokines in plaque tissue and might be possible plasma markers for a vulnerable atherosclerotic disease. We thereby propose that these cytokines can be used as surrogate markers for the identification of patients with high-risk plaques.

Introduction

Most acute cardiovascular events are due to thrombotic occlusion caused by ruptured atherosclerotic plaques.^{1,2} Plaque rupture is mainly caused by an inflammatory degradation of the plaque connective tissue, most importantly the fibrous cap.³⁻⁶ Atherosclerotic plaques that are at high risk of rupturing are often referred to as vulnerable plaques.⁷ Such plaques are characterized by abundant inflammation, a large core of lipids and necrotic cells, and a thin fibrous cap.

The identification of vulnerable atherosclerotic plaques in patients poses a significant clinical challenge. Although advanced imaging technologies such as intravascular ultrasound (IVUS) and magnetic resonance imaging or CT-positron emission tomography (CT-PET) using radiolabeled glucose have been applied to study efficacy in cardiovascular intervention trials,⁸⁻¹⁰ the wider use of these technologies is limited by invasiveness risks, possible contrast need, high cost and/or the dependence on radiation. An alternative or complementary approach to the use of imaging techniques to identify vulnerable plaques relies on the use of circulating biomarkers. A circulating-biomarker-approach could also enable the assessment of the arterial system systemically rather than focusing on a restricted arterial segment as is the case with current imaging techniques. Previous studies have reported high-sensitivity C-reactive protein (hsCRP) as a possible biomarker of vulnerable plaque; hsCRP is a well-established biomarker of cardiovascular risk and its circulating levels have been shown to correlate with carotid intima-media thickness (IMT).¹¹⁻¹³ However, although chronic arterial inflammation could be reflected by a low-grade elevation of plasma hsCRP, we have recently shown that there is no association between plasma hsCRP levels and carotid plaque inflammation.¹⁴ Suitable biomarkers for the identification of high-risk plaques in patients are therefore currently lacking.

Here, we propose that the plasma level of certain pro-inflammatory cytokines can be used as surrogate biomarkers for the identification of patients with high-risk plaques. We postulated that abundant cytokines within atherosclerotic plaques may diffuse into the circulation and thereby that plasma levels of such cytokines could reflect the inflammatory activity in the plaques. Plasma levels of several cytokines have been shown to correlate with the progression of the atherosclerotic disease or have been considered as markers of future cardiovascular events.¹⁵

To test this notion, we have analyzed the levels of several cytokines in both atherosclerotic plaques and plasma from the same patients with symptomatic and asymptomatic carotid disease. We show for the first time that several of the cytokines expressed in the plaque tissue correlate with plasma levels of the same cytokines. Plasma levels of MIP-1 β , fractalkine and TNF- α also correlate with several other of the pro-inflammatory cytokines in the plaque tissue and high plasma levels of these three cytokines may thus mark the presence of high grade inflammatory plaques. Furthermore, high plasma levels of these three cytokines helps in predicting the occurrence of postoperative transient ischemic attacks (TIAs).

Material and Methods

Patient characteristics

We collected 200 human carotid plaques from 197 patients (three of them treated bilaterally). Carotid endarterectomy indications were plaques associated with ipsilateral symptoms (n=105, stroke, TIA or amaurosis fugax) and stenosis of >70%, measured by duplex, or plaques not associated with symptoms and stenosis of >80% (n=95). All patients were evaluated by a neurologist prior to the operation. Informed consent was given by each patient. Venous blood samples (EDTA-plasma) were collected the day before surgery. Total cholesterol, triglycerides, high-density lipoprotein (HDL) and low-density lipoprotein (LDL) cholesterol, white blood cell counts (WBC), hsCRP and creatinin were determined by routine laboratory procedures. Age, hypertension, dyslipidemia, use of statins, past or current smoking and diabetes were recorded based on medical record. Three patients underwent a second endarterectomy on the contralateral side. The study was approved by the local ethical committee and performed according to the declaration of Helsinki.

Plaque preparation and histology

Plaques were snap-frozen in liquid nitrogen immediately after surgical removal. All plaque tissue, except for a 1 mm fragment kept for histology, was weighed, cut into pieces while still frozen, and homogenized as previously described.¹⁶

For histology, 1 mm fragments from the most stenotic region were taken and cryosectioned in sections of 8µm. The sections were fixed with Histochoice (Amresco, Ohio, USA), dipped in 60% isopropanol and then in 0.4% Oil Red O in 60% isopropanol (for 20 min) to stain lipids. Vascular smooth muscle cells were stained using a (alpha-actin) primary antibody monoclonal mouse anti-human smooth muscle actin clone 1A4 (DakoCytomation, Glostrup, Denmark), diluted in 10% rabbit serum 1:50, and secondary antibody biotin rabbit anti-mouse Ig

(DakoCytomation, Glostrup, Denmark), dilution 1:200 in 10% of rabbit serum. When staining for macrophages primary antibody monoclonal mouse anti-human CD68 (DakoCytomation, Glostrup, Denmark), diluted in 10% rabbit serum 1:100, and secondary antibody polyclonal rabbit anti-mouse (DakoCytomation, Glostrup, Denmark), dilution 1:200 in 10% of rabbit serum, were used. When staining for Fractalkine a mouse anti human monoclonal antibody was used (Anti-CX3CL1 antibody, Abcam, Cambridge, UK) and when staining for TNF- α a mouse anti human monoclonal antibody was used (Anti-TNF alpha antibody, Abcam, Cambridge, UK). The procedures were performed according to the manufacturers instructions. Stained plaque areas (% of plaque area) were quantified blindly using BiopixiQ 2.1.8 (Gothenburg, Sweden) after scanning with ScanScope Console Version 8.2 (LRI imaging AB, Vista CA, USA).

Cytokine assessment

Plaque homogenate levels and plasma levels of cytokines (fractalkine, interferon- γ (IFN- γ), IL-6, MCP-1, MIP-1 β , platelet-derived growth factor-AB/BB (PDGF-AB/BB), RANTES, s-CD40L, vascular endothelial growth factor (VEGF) and TNF- α) were analysed using Luminex technology. The procedure was performed according to the manufacturer's instructions (Human Cytokine/chemokine immunoassay, Millipore Corporation, MA, USA) and analysed with Luminex 100 IS 2.3 (Austin, Texas, USA). The EDTA plasma was centrifuged for 10 minutes, 600 x G in room temperature. The levels PDGF, RANTES, s-CD40L and VEGF were found to be higher in plasma than in plaque tissue. As we could not exclude that this was due to a release of these factors from platelets during plasma preparation we did not include them in further analyses.

Clinical follow up

The Swedish National in-patient Health Register was analysed in order to identify postoperative neurological ischemic (non-haemorrhagic) events corresponding to the following codes of the 10th revision of the International Classification of Diseases (ICD-10): G45, G46 and I63. This is a nation-wide validated register where more than 99 percent of all somatic and psychiatric hospital discharges are registered.¹⁷

In doubtful cases, information was checked through telephone interviews and review of the medical charts. All deaths were verified against the Swedish National Population Register.

Definition of outcomes

All neurological ischemic events (stroke, TIA, amaurosis fugax) were registered. Patients suffering more than one episode were classified as suffering multiple events and only the first chronological event was used in the survival analysis.

Statistics

Cytokines were non-normally distributed. All measurements of the plaque were normalized against plaque wet weight or presented as total plaque content of respective cytokine. Mann-Whitney or Students't-test was used for two-group comparisons as appropriate. For categorical data χ^2 test was used. For correlation analysis, Spearman's rho was used. Follow-up data were available for 189 patients. Kaplan-Meier survival analysis was used to test the statistical significance of differences in the absence of postoperative events during follow up. Cox proportional hazard regression model was used to correct for interferences. Associations shown in supplemental table 1 were adjusted for confounding factors using linear regression analysis. SPSS 21.0 was used for statistical analysis. Values are presented as mean and standard

deviation (SD) or hazard ratio (HR) with 95% confidence intervals (CI). $p < 0.05$ was considered significant.

Results

We included 105 symptomatic plaques and 95 asymptomatic plaques obtained from 197 patients in the current analysis. The mean time between the clinical event and surgery was 15.1 ± 8.4 days for patients with a symptomatic plaque. The clinical characteristics of the study cohort are shown in Table 1.

Cytokine levels in plaque homogenates and in plasma

The most abundant pro-inflammatory cytokines in the plaques were fractalkine, MCP-1 and IL-6 (data not shown). The concentration of fractalkine and IL-6 was about 1 ng per g of plaque tissue, while the concentration of MCP-1 was around 3 ng/g plaque tissue. TNF- α , MIP-1 β and IFN- γ were detected at concentrations of around 200 pg/g plaque tissue or below.

Association between plaque and plasma cytokine levels

Next we investigated whether cytokines secreted in the plaques can serve as markers of plaque inflammation. We found significant correlations between the plaque and plasma levels of MIP-1 β , TNF- α , IFN- γ and fractalkine (Table 2). The same pattern was found for fractalkine, MIP-1 β and TNF- α , even though TNF- α did not reach statistical significance ($p=0.054$) when adjusting for age, gender, smoking, diabetes and medications (statins and betablockers) (Supplementary Table I). Since it is likely that the associations between plaque and circulating cytokines can be affected by the occurrence of an acute clinical event (both by inducing systemic inflammation and by activation of repair responses in the plaque) we also analyzed symptomatic and asymptomatic plaques separately. In line with our hypothesis, the associations between plaque and plasma cytokines were stronger in patients with asymptomatic plaques (Table 2). It could also be argued that if plasma cytokines reflect the

inflammatory activity of the removed carotid plaque associations would be stronger for the total plaque content than for the content normalized against plaque weight. However, the same pattern of associations was observed when correlating against the total plaque content of cytokines (Supplementary Table II).

Plasma cytokines as markers of plaque inflammation

We next analyzed whether any of the circulating cytokines could function as general markers of plaque inflammation. We found that the plasma levels of MIP-1 β , fractalkine, TNF- α and IFN- γ all demonstrated significant associations with the expression of most pro-inflammatory cytokines in plaques (Table 3). Again, these correlations were found to be more significant in patients with asymptomatic plaques than in patients with symptomatic plaques (Supplementary Tables III A and B). Taken together these findings show that pro-inflammatory cytokines, particularly MIP-1 β , TNF- α and fractalkine, measured in plasma reflect plaque expression of pro-inflammatory cytokines.

Correlations between plasma and plaque cytokine levels with plaque histology

Next, we analyzed if cytokine levels were associated with more traditional markers of plaque inflammation by determining macrophage staining (CD68) histologically. We also included measurements for smooth muscle cells (α -actin) and lipids (Oil Red O). We observed significant associations between the abundance of plaque macrophages and plaque levels of MIP-1 β and MCP-1 (Table 4). Plaque lipid staining was significantly correlated with plaque levels MIP-1 β , MCP-1 and IL-6, whereas plaque α -actin staining correlated inversely with MIP-1 β and IL-6 (Table 4). These associations were similar in asymptomatic and symptomatic patients (data not shown). To confirm the presence and to locate the inflammatory cytokines within the plaque tissue additional staining's for TNF- α and

fractalkine were performed (Figure 1). We also found a significant inverse correlation between plasma levels of fractalkine and the plaque α -actin staining ($r=-0.164$, $P=0.022$).

A plaque inflammation score based on the plaque and plasma levels of cytokines tested

To determine whether the levels of plasma cytokines could identify the presence of plaques with high inflammation, we created a plaque inflammation score. This score is based on tertiles of the plaque levels of the cytokines that were increased in plaque tissue and showed correlations with the corresponding plasma levels. The score was obtained by adding the tertiles for the 4 identified cytokines (MIP-1 β , TNF- α , IFN- γ and fractalkine) and varied between 4 and 12, where a score of 12 represents plaques with the highest inflammation. The strongest correlations with the plaque inflammation score were found for plasma MIP-1 β , TNF- α and fractalkine ($r=0.51$, $p<0.000001$, $r=0.49$, $p<0.000001$ and $r=0.46$, $p<0.000001$, respectively). The plaques were then classified as having high inflammation and low inflammation depending if they were above or below the median inflammation score. Plasma TNF- α levels in the upper tertile pointed to the presence of lesions with high inflammation with sensitivity of 67.7% and specificity of 83.3%, the upper tertile of MIP-1 β with sensitivity of 65.2% and specificity of 78.5% and the upper tertile of fractalkine with a sensitivity of 65.6% and a specificity of 81.3%. These findings show that high plasma levels of MIP-1 β , TNF- α and fractalkine are associated with a higher plaque content of pro-inflammatory cytokines and these three cytokines could **identify** a plaque with a high inflammatory grade.

Plasma cytokines and postoperative cerebrovascular events

Finally we examined if plasma levels of the measured cytokines could predict a future cerebrovascular (ischemic) event. After a mean follow-up time of 60.0 +/- 21.6 months, 25 patients had suffered a cerebrovascular event. Patients in the upper tertile of the three plasma

cytokines which had shown the strongest association to plaque inflammation (i.e. MIP-1 β , TNF- α and fractalkine) had an increased incidence of contralateral TIAs as shown by Kaplan–Meier curves of event-free survival (Figure 2) when compared to lowest and middle tertile. The increased risk for future TIAs remained significant after controlling for age, sex, diabetes, smoking and the use of statins or betablockers in a Cox Proportional Hazard model (MIP-1 β HR 12.6, 95% C.I. 1.2-132.2, p=0.034; TNF- α HR 10.9, 95% C.I. 1.1-113.9 p=0.046; fractalkine HR 12.7, 95% C.I. 1.2-136.6 p=0.036).

Discussion

Differences in plasma and plaque cytokine levels

The present study highlights, for the first time, significant associations between several pro-inflammatory cytokines that are abundant in atherosclerotic plaques and the levels of these cytokines in the plasma. A likely explanation for the observed associations is that cytokines diffuse from atherosclerotic lesions out into the circulation thus they could ultimately serve as markers of plaque inflammation. Of note, the carotid lesions used for analysis of plaque cytokines in this study are expected to represent only a minor part of the total plaque burden of the patient. It seems unlikely that such a small tissue could release enough cytokine levels to fully explain the associations we observed. One likely explanation to the associations between plasma and carotid plaque cytokines could be that the inflammatory levels in the latter is representative of the level of inflammation in plaques in other parts of the arterial tree. Furthermore, the levels of circulating plasma cytokines might be influenced by other concomitant conditions. In line with this, the associations between plaque and plasma cytokines were found to be stronger in patients with asymptomatic plaques than in patients with symptomatic plaques in whom the recent cardiovascular insult will have induced a systemic inflammatory response.¹⁸⁻²⁰ It has previously been shown that the protein profile of the atherosclerotic plaque differs depending on the morphology of the plaque. Plaques with a vulnerable phenotype and intraplaque hemorrhage have higher levels of inflammatory markers.²¹ This suggests that acute complications such as a plaque rupture or intra-plaque hemorrhage are associated with local activation of inflammation and that this could be reflected in the circulation.

Plasma cytokine levels as markers of plaque inflammation

The plasma levels of MIP-1 β , TNF- α , IFN- γ and fractalkine were found not only to correlate with their respective abundance in carotid plaques but also with the abundance of several other cytokines in the plaques. The possibility that circulating MIP-1 β , TNF- α , IFN- γ and fractalkine can be used as surrogate marker for plaque expression of these cytokines is of potential clinical relevance because all of these factors have been shown to affect atherosclerotic plaque development in experimental studies.^{22,23} Moreover, plasma levels of MIP-1 β , TNF- α , IFN- γ and fractalkine have all been suggested as markers of CVD risk and severity. Plasma levels of MIP-1 β were recently shown to predict future cardiac events in patients with intermediate coronary artery lesions.²⁴ Plasma TNF- α levels have been shown to correlate with carotid plaque burden in healthy men and were associated with increased risk of recurrent myocardial infarction in the Cholesterol And Recurrent Events (CARE) study.²⁵ Fractalkine and its receptor have also been shown to be present in human atherosclerotic plaques.^{27, 28} Furthermore, increased plasma levels of fractalkine have been reported in patients with CVD, including patients with unstable angina or verified plaque rupture.^{29, 30} IFN- γ has been considered to have an important role in atherosclerosis, but its use as a circulating biomarker has been limited by the rapid neutralization following its' release. However, there are studies showing enhanced expression of IFN- γ in circulating cells of patients with coronary artery disease and acute coronary syndromes.^{31,32} Increased plasma levels of IFN- γ have been found in male patients with coronary artery disease.³³

For two of the most abundant plaque cytokines, IL-6 and MCP-1, we did not observe any associations with the corresponding plasma levels and we only found some weak correlations between IL-6 with the abundance of some pro-inflammatory cytokines in plaque tissue. No such associations were found for MCP-1. The reason for the poor association between plaque and plasma levels for these two cytokines remains to be elucidated. However, a possible

explanation would be the biological sources of cytokines as MCP-1 and IL-6. As the adipose tissue is known to be rich in inflammatory cytokines as IL-6 and MCP-1, an enhanced release into the circulation would then affect the associations seen between plasma and plaque levels of cytokines. This enhanced release may still predict a high risk individual but does not necessary need to reflect the inflammatory activity in the vascular wall. It is also possible that there exist differences in the way cytokines are trapped and metabolized locally in the plaque that could explain this lack of association between plaque and plasma levels.

Cytokines as potential biomarkers

An important question is whether the association between plasma and plaque cytokines identified in the present study has clinical value and can be used to identify subjects with high-risk lesions in the clinical practice. Inflammation is considered to have an important role in plaque rupture, which indicates that circulating biomarkers of plaque inflammation could be of clinical importance. We developed a score based on the plaque abundance of MIP-1 β , TNF- α , IFN- γ and fractalkine, which can be used as a general measure of plaque inflammation. High plasma levels (upper tertile) of MIP-1 β , TNF- α , and fractalkine identified the presence of a plaque with high inflammation (above median) with a sensitivity between 65 and 67% and a specificity between 78 and 83%. Thus, measurements of these cytokines could provide important supportive information when used in combination with imaging techniques such as CT-angiography, IVUS, CT-PET and magnetic resonance imaging.

This theory is supported by the finding that patients with plasma levels of MIP-1 β , TNF- α and fractalkine within the highest tertile also had an increased risk of cerebrovascular events at follow-up.

It is also possible that changes in the plasma levels of these cytokines can be used as surrogate markers to assess the effect on plaque inflammation in cardiovascular intervention studies. However, this possibility needs to be assessed in future clinical studies.

Study limitations

Our study has certain limitations. We used two different approaches to assess plaque inflammation; the cytokine content of plaque homogenates and histologic evaluation of plaque sections. Plasma levels of MIP-1 β , TNF- α , IFN- γ and fractalkine correlated significantly with most plaque cytokines but not with the presence of plaque lipids, macrophages and VSMCs in plaque sections. Moreover, although the plaque content of several cytokines showed significant association with plaque lipids and macrophages as well as inverse associations with VSMCs, these correlations were not very strong. These findings challenge the approaches used in terms of which approach provides the most accurate estimate of plaque inflammation. Measurement of cytokines in plaque homogenates has the advantage of providing information of the inflammatory activity in almost the entire lesion. This is important as plaques are heterogeneous in nature usually containing both areas that are relatively fibrous and others with more extensive inflammation and cell death. A limitation with this approach is the difficulty in determining the level of cytokine recovery in the extraction procedure as well as the possible influence of any binding proteins that may confound the analysis.

Histological analysis of plaque sections is a well-established method for analysis of plaque vulnerability and the definition of a vulnerable plaque is based on its morphological characteristics. However, this approach is mostly based on sections from a single segment of the plaque and does not consider the heterogeneity of the plaque tissue. In the current study,

we used a one-mm section from the most stenotic part of the lesion for histological analysis. Although this provides a way to standardize the analysis it might give a misleading estimate of plaque inflammation, as this often is more pronounced in more proximal parts of the lesion.³⁴ Moreover, there is accumulating evidence of the existence of multiple macrophage subsets. The classic staining for macrophages with CD68 antibodies includes some with a non-inflammatory, repair phenotype, too. Therefore, general staining for macrophages alone may not provide accurate information about the inflammatory state of the lesion.

The results of our analysis on longitudinal data need to be confirmed in larger cohorts with longer observational intervals. Furthermore, we lack data regarding changes in cytokines levels during follow-up.

Finally, the use of a plaque inflammation score based only on the abundance of those cytokines for which significant associations with the respective plasma levels had been identified could lead to an overestimation of the ability of plasma MIP-1 β , TNF- α , and fractalkine to predict the presence of inflamed plaques. However, the associations between the plasma levels of these cytokines and the plaque inflammation score was similar or only weakly stronger than for the individual expression of most plaque cytokines.

Conclusions

Our observations demonstrate that the plasma levels of MIP-1 β , TNF- α , and fractalkine reflect the abundance of several pro-inflammatory and atherogenic cytokines in plaque tissue and can be used to identify the presence of plaques with high inflammation. We also provide evidence for MIP-1 β , TNF- α , and fractalkine as possible markers predicting future TIAs, strengthening the role of the cytokines as markers of atherosclerotic vulnerability. Analysis of

the plasma levels of these cytokines combined with plaque imaging techniques might help to identify subjects with high-risk lesions. Such biomarkers can also be used to assess the response to treatments aiming at decreasing atherosclerotic plaque inflammation.

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Conflict of interest

None declare.

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Figure 1

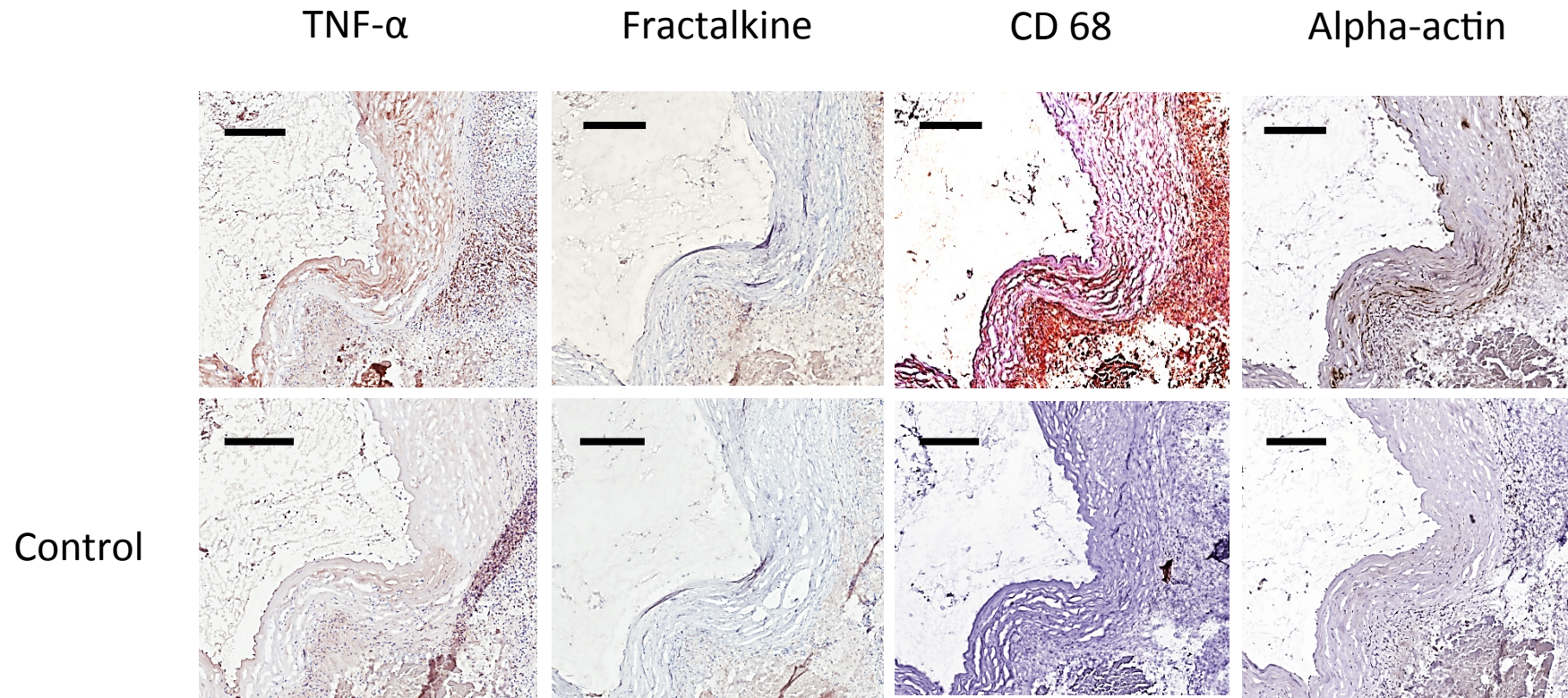


Figure 2. Sections of human carotid plaques stained for TNF- α , fractalkine, macrophages (CD68), vascular smooth muscle cells (alpha-actin) and respective control. Scale bars 300 μ m.

Figure 2

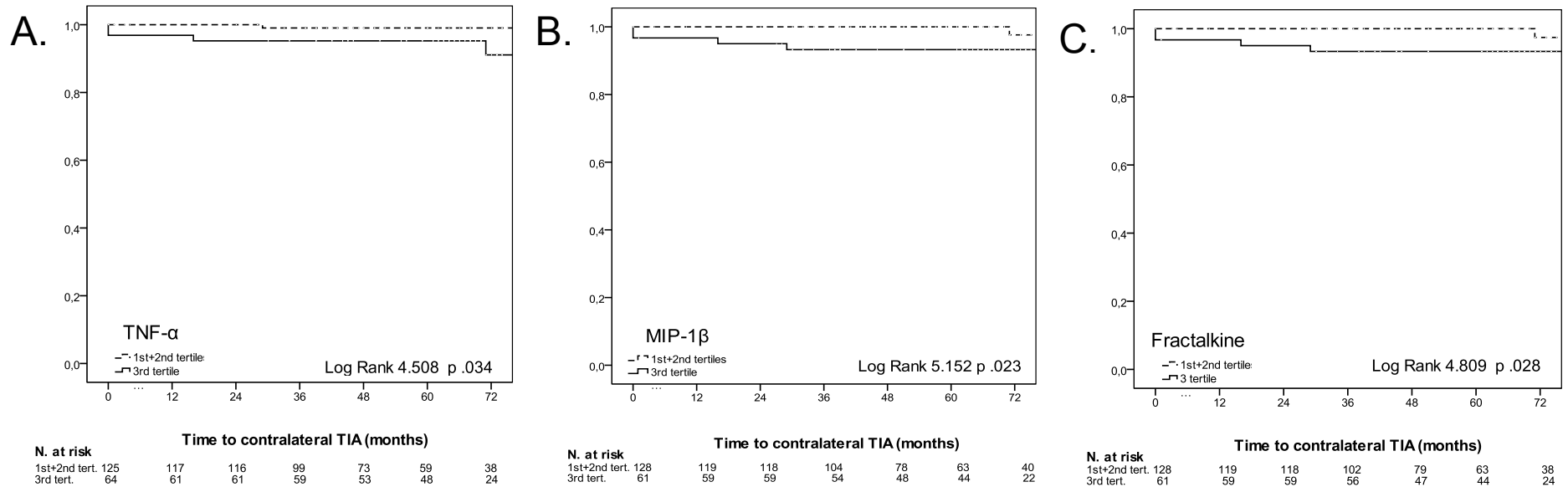


Figure 1. Kaplan-Meier survival analysis. Time to postoperative transient ischemic attack expressed in months. Patients are grouped based on tertiles of plasma levels of A) TNF- α B) MIP-1 β and C) fractalkine (1st+2nd tertiles vs 3rd tertile). N. at risk: number of patients at risk at each time interval.

Table 1. *Clinical characteristics of the patients (n=200).*

	<i>All</i>	<i>AS</i>	<i>S</i>	<i>P</i>
Age (years)	69.4 (SD 8.2)	66.9 (SD 6.7)	71.2 (SD 8.8)	<0.001
Diabetes (%)	33.5% (n=66)	30.1% (n=22)	42% (n=44)	<0.01
Hypertension (%)	78% (n=153)	80% (n=76)	73% (n=77)	NS
Smoking (past or current, %)	82% (n=163)	84% (n=80)	79% (n=83)	NS
Dyslipidemia (%)	94% (n=188)	98% (n=93)	90% (n=95)	NS
Statin treatment (%)	87% (n=173)	92% (n=87)	82% (n=86)	<0.05
Fasting Lipoproteins (mmol/L):				
Total cholesterol	4.4 (SD 1.1)	4.3 (SD 1)	4.4 (SD 1.2)	NS
LDL cholesterol	2.4 (IQR 1.9-3.1)	2.2 (IQR 1.8-3.1)	2.5 (IQR 2.0-3.3)	NS
HDL cholesterol	1.1 (IQR 0.9-1.3)	1.1 (IQR 0.9-1.4)	1.1 (IQR 0.9-1.2)	NS
Triglycerides	1.3 (IQR 0.9-1.8)	1.3 (IQR 0.9-1.8)	1.2 (IQR 0.9-1.7)	NS
Creatinin (mmol/L)	89.6 (SD 24.1)	87 (SD 24.4)	92 (SD 23.6)	NS
High sensitive-CRP (mg/L)	4.0 (IQR 2.0-6.7)	3.8 (IQR 1.9-5.9)	4.2 (IQR 2.1-7.2)	NS
White blood cell count (10 ⁹ /L)	7.9 (SD 2)	7.9 (SD 1.9)	7.9 (SD 2.1)	NS

All, all patients; AS, asymptomatic patients; S, symptomatic patients. NS, non-significant. P for significant differences comparing symptomatic and asymptomatic patients. Normally distributed variables are presented as mean and standard deviation (SD) and non-normally distributed variables are presented as median and inter quartile range (IQR).

Table 2. Correlations between respective plaque (pg/g) and plasma cytokines (pg/mL).

Cytokine	<i>All</i>	<i>S</i>	<i>AS</i>
IL-6	NS	NS	NS
MCP-1	NS	NS	NS
MIP-1 β	r=0.276 ***	r=0.217 *	r=0.315 ***
TNF- α	r=0.290 ***	r=0.197 *	r=0.376 ***
IFN- γ	r=0.306 ***	NS	r=0.444 ***
Fractalkine	r=0.468 ***	r=0.358 ***	r=0.590 ***

Correlations between plasma and plaque levels of respective cytokines in all patients (*All*), symptomatic patients (*S*) and asymptomatic patients (*AS*). Interleukin-6, IL-6. Monocyte chemoattractant protein-1, MCP-1. Macrophage inflammatory protein-1 β , MIP-1 β . Tumor necrosis factor- α , TNF- α . Interferon- γ , IFN- γ . Significance marked by * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.005$. NS, non-significant.

Table 3. Correlations between plaque (P; pg/g) and plasma (B; pg/mL) cytokines in all patients.

Cytokine	B-IL-6	B-MCP-1	B-MIP-1 β	B-TNF- α	B-IFN- γ	B-Fractalkine
P-IL-6	NS	NS	r=0.204***	r=0.228 ***	NS	r=0.219 ***
P-MCP-1	NS	NS	NS	NS	NS	NS
P-MIP-1 β	NS	NS	r=0.276 ***	r=0.263 ***	r=0.155 *	r=0.276 ***
P-TNF- α	r=0.159*	NS	r=0.300 ***	r=0.290 ***	r=0.214 ***	r=0.247 ***
P-IFN- γ	NS	NS	r=0.394 ***	r=0.402 ***	r=0.306 ***	r=0.326 ***
P-Fractalkine	r=0.183*	NS	r=0.508 ***	r=0.499 ***	r=0.328 ***	r=0.468 ***

Interleukin-6, IL-6. Monocyte chemoattractant protein-1, MCP-1. Macrophage inflammatory protein-1 β , MIP-1 β .

Tumor necrosis factor- α , TNF- α . Interferon- γ , IFN- γ Significance marked by * P<0.05, ** P<0.01 and *** P<0.005.

NS, non-significant.

Table 4. Correlations between plaque cytokines (pg/g) and histological plaque features (% area).

	<i>Lipids (ORO)</i>	<i>VSMCs (α-actin)</i>	<i>Macrophages (CD68)</i>
IL-6	r=0.298***	r=-0.146*	NS
MCP-1	r=0.332***	NS	r=0.215***
MIP-1 β	r=0.259***	r=-0.282***	r=0.145*
TNF- α	NS	NS	NS
IFN- γ	NS	NS	NS
Fractalkine	NS	NS	NS

Interleukin-6, IL-6. Monocyte chemoattractant protein-1, MCP-1. Macrophage inflammatory protein-1 β , MIP-1 β . Tumor necrosis factor- α , TNF- α . Interferon- γ , IFN- γ Vascular smooth muscle cells, VSMCs. Significance marked by * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.005$. NS, non-significant.

Supplemental Tables

Supplementary table I. *Linear regression analysis between plaque and plasma levels of respective cytokine after correction for age, gender, diabetes, smoking and the use of statins and betablockers. All patients (All), symptomatic patients (S) and asymptomatic patients (AS).*

Cytokine	<i>All</i>	<i>S</i>	<i>AS</i>
IL-6	NS	NS	NS
MCP-1	NS	NS	NS
MIP-1 β	$\beta=0.18$ *	$\beta=0.23$ *	NS
TNF- α	NS	NS	NS
IFN- γ	NS	NS	NS
Fractalkine	$\beta=0.17$ *	$\beta=0.21$ *	NS

Interleukin-6, IL-6. Monocyte chemoattractant protein-1, MCP-1. Macrophage inflammatory protein 1- β , MIP-1 β . Tumor necrosis factor- α , TNF- α . Interferon- γ , IFN- γ . Significance marked by * P<0.05, NS, non-significant.

Supplementary table II. *Correlations between total plaque abundance of respective plaque cytokine (pg/g) and plasma cytokines (pg/ml) in all patients (All), symptomatic patients (S) and asymptomatic patients (AS).*

Plaque/Plasma Cytokine	All	S	AS
IL-6	NS	NS	NS
MCP-1	NS	NS	NS
MIP-1 β	r=0.220 ***	NS	r=0.273 **
TNF- α	r=0.293 ***	r=0.251 *	r=0.330 ***
IFN- γ	r=0.304 ***	NS	r=0.397 ***
Fractalkine	r=0.495 ***	r=0.441 ***	r=0.571 ***

Interleukin-6, IL-6. Monocyte chemoattractant protein-1, MCP-1. Macrophage inflammatory protein 1- β , MIP-1 β . Tumor necrosis factor- α , TNF- α . Interferon- γ , IFN- γ . Significance marked by * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.005$. NS, non-significant.

Supplementary table III A. *Correlations between respective plaque (P) and plasma (B) cytokines in the symptomatic patients. Plasma levels presented as pg/mL and plaque levels as pg/g.*

Cytokine	B-IL-6	B-MCP-1	B-MIP-1 β	B-TNF- α	B-IFN- γ	B-Fractalkine
P-IL-6	NS	NS	NS	r=0.205 *	NS	NS
P-MCP-1	NS	NS	NS	NS	NS	NS
P-MIP-1 β	NS	NS	r=0.217 *	r=0.206 *	NS	r=0.264 **
P-TNF- α	NS	NS	NS	r=0.197 *	NS	NS
P-IFN- γ	NS	NS	r=0.271 **	r=0.245 *	NS	r=0.209 *
P-Fractalkine	r=0.230 *	NS	r=0.428 ***	r=0.381 ***	r=0.213 *	r=0.358 ***

Interleukin-6, IL-6. Monocyte chemoattractant protein-1, MCP-1. Macrophage inflammatory protein 1- β , MIP-1 β .

Tumor necrosis factor- α , TNF- α . Interferon- γ , IFN- γ . Significance marked by * $P<0.05$, ** $P<0.01$ and ***

$P<0.005$. NS, non-significant.

Supplementary table III B. *Correlations between respective plaque (P) and plasma (B) cytokines in the asymptomatic patients. Plasma levels presented as pg/mL and plaque levels as pg/g.*

Cytokine	B-IL-6	B-MCP-1	B-MIP-1 β	B-TNF- α	B-IFN- γ	B-Fractalkine
P-IL-6	NS	NS	r=0.323 ***	r=0.226 *	NS	r=0.302 ***
P-MCP-1	NS	NS	NS	NS	NS	NS
P-MIP-1 β	NS	NS	r=0.315 ***	r=0.300 ***	r=0.207 *	r=0.317 ***
P-TNF- α	r=0.244 *	NS	r=0.398 ***	r=0.376 ***	r=0.307 ***	r=0.349 ***
P-IFN- γ	NS	NS	r=0.502 ***	r=0.549 ***	r=0.444 ***	r=0.455 ***
P-Fractalkine	NS	NS	r=0.590 ***	r=0.629 ***	r=0.434 ***	r=0.590 ***

Interleukin-6, IL-6. Monocyte chemoattractant protein-1, MCP-1. Macrophage inflammatory protein 1- β , MIP-1 β .

Tumor necrosis factor- α , TNF- α . Interferon- γ , IFN- γ . Significance marked by * $P<0.05$, ** $P<0.01$ and *** $P<0.005$.

NS, non-significant.