Monocyte-chemoattractant protein-1 Levels in Human Atherosclerotic Lesions Associate with Plaque Vulnerability

Running title: *Georgakis et al.; MCP-1 in human plaque vulnerability*

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Abstract

Objective: To determine whether MCP-1 levels in human atherosclerotic plaques associate with plaque vulnerability features.

Approach and Results: We measured MCP-1 levels in human atherosclerotic plaque samples from 1,199 patients in the Athero-EXPRESS Biobank who underwent endarterectomy for treatment of carotid stenosis. We explored associations with histopathological and molecular features of plaque vulnerability, clinical plaque manifestations, and vascular events up to 3 years after endarterectomy. Following adjustments for age, sex, and vascular risk factors, MCP-1 plaque levels were associated with histopathological markers of plaque vulnerability (large lipid core, low collagen content, high macrophage burden, low smooth muscle cell burden, intraplaque hemorrhage) and with a composite vulnerability index (range 0-5, beta per SD-increment in MCP-1: 0.42, 95%CI: 0.30-0.53, $p=5.4x10^{-13}$). We further found significant associations with higher plaque levels of other chemokines and pro-inflammatory molecules, and markers of neovascularization and matrix turnover. When exploring clinical plaque instability, MCP-1 plaque levels were higher among individuals with symptomatic plaques as compared to those with asymptomatic plaques (OR per SD-increment in MCP-1: 1.36, 95%CI: 1.09-1.69). MCP-1 levels were further associated with a higher risk of periprocedural major adverse vascular events and strokes occurring in the first 30 days after plaque removal.

Conclusions: Higher MCP-1 plaque levels are associated with histopathological, molecular, and clinical hallmarks of plaque vulnerability in individuals undergoing carotid endarterectomy. Our findings highlight a role of MCP-1 in clinical plaque instability in humans and complement previous epidemiological, genetic, and experimental studies supporting the translational perspective of targeting MCP-1 signaling in atherosclerosis.

Keywords: MCP-1; CCL2; atherosclerosis; inflammation; cardiovascular disease; plaque vulnerability; macrophages.

Abbreviations and acronyms

MCP-1	monocyte chemoattractant protein-1
IL1β	interleukin-1β
IL6	interleukin-6
CCR2	CC-chemokine receptor 2
CCL2	CC-chemokine ligand 2
SMC	smooth muscle cell
LDL-C	low-density lipoprotein cholesterol
eGFR	estimated glomerular filtration rate
BMI	body mass index
FDR	false discovery rate

Introduction

Inflammatory mechanisms are critically involved in the pathogenesis of atherosclerosis.[1, 2] Recent clinical trials on cardiovascular prevention in patients with symptomatic atherosclerotic disease have demonstrated a benefit of anti-inflammatory treatment on top of standard therapy.[3-5] The differences in the efficacy of the tested drugs in these trials risk (canakinumab, methotrexate, colchicine) to lower vascular also highlighted the importance of targeting specific inflammatory pathways.[3, 5-7] So far, translational efforts mostly focused on the inflammasome- IL1β- IL6-axis.[8] Yet, experimental and genetic studies place emphasis on pro-inflammatory mechanisms in atherosclerosis beyond this axis, as has specifically been shown for the chemokine system.[7, 9]

Monocyte chemoattractant protein-1 (MCP-1, also called CC-chemokine ligand 2), is a CC family chemokine that mobilized monocytes from the bone marrow and attracts them to sites of inflammation[10] including the atherogenic arterial wall.[11-14] Mice lacking MCP-1 or its receptor CCR2 are protected from atherosclerosis and pharmacological inhibition of the MCP-1/CCR2 axis reduces plaque size in experimental atherosclerosis.[15-20] Recent genetic and observational data from humans further support associations of circulating MCP-1 levels with the risk of stroke and coronary artery disease.[21-23] Yet, the translational potential of targeting the MCP-1/CCR2 pathway in human atherosclerosis and specifically whether MCP-1 activity within human plaques is causally involved in atheroprogression remains elusive. To determine the potential clinical utility of targeting MCP-1, it would be critical to clarify associations between MCP-1 levels within plaques and features of plaque vulnerability and instability that underlie the occurrence of clinical events including stroke and myocardial infarction.

Here, we analyzed carotid plaque samples from >1000 individuals, who underwent endarterectomy for treatment of asymptomatic or symptomatic carotid stenosis. Our aims were to explore associations of plaque MCP-1 levels with: (i) histopathological features of plaque vulnerability (lipid core, collagen content, macrophage burden, smooth muscle cell (SMC) burden, intraplaque hemorrhage); (ii) plaque inflammation and matrix turnover as assessed by

the plaque levels of inflammatory cytokines and metalloproteinase activity; (iii) clinical plaque instability, as defined by a symptomatic plaque causing an acute cerebrovascular event; and (iv) major adverse vascular events occurring after plaque removal (**Figure 1A**).

Materials and Methods

The datasets from Athero-Express analyzed for the current study are available upon reasonable request and application to Athero-Express Biobank Study through a Data Transfer Agreement due to consent restrictions and local regulations. Codes used for this study are available here: https://github.com/swvanderlaan/2020_georgakis_vanderlaan_MCP1.

Study population

We used data from the Athero-Express Biobank (http://www.atheroexpress.nl), an ongoing prospective study of patients undergoing endarterectomy for manifestations of atherosclerosis.[24] Carotid endarterectomy was performed following recommendations by the Asymptomatic Carotid Atherosclerosis Study (ACAS)[25] and The North American Symptomatic Carotid Endarterectomy Trial (NASCET).[26] Patients were recruited from the St. Antonius Hospital Nieuwegein and University Medical Center Utrecht in Utrecht, Netherlands between 2002 and 2019. Individuals who agreed to participate completed questionnaires about medical history and medication use prior to the operation and provided blood samples for biochemical and hematological analyses. Their plaque samples were post-operatively collected and analyzed as described below. Individuals were included in the current study on the basis of having undergone carotid endarterectomy and having available measurements of MCP-1 levels in plaque (**Figure 1**). The study protocol conforms to the Declaration of Helsinki and was approved by the ethics committee on research on humans of the University Medical Center Utrecht. All participants provided written informed consent.

Histopathological analysis of atherosclerotic plaque composition

Following carotid endarterectomy, plaque samples were immediately transferred to the laboratory. Plaques were divided in parallel segments of 5-mm thickness perpendicular to the arterial axis and the segment with the greatest plaque burden was subjected to histopathological examination, as previously described.[27-29] All stained sections were examined microscopically and digitally stored. For the purposes of the current study, we explored five plaque traits that are established hallmarks of plaque vulnerability: lipid content, collagen deposition, macrophages, smooth muscle cells, and intraplaque hemorrhage.[30, 31] Two independent observers manually scored stainings for these traits using previously defined semi-quantitative methods.[24, 27-29] In brief, plaque lipid content was quantified visually as a percentage of fat deposition to total plaque area with the use of hematoxylin-eosin and picrosirius red stains; a large lipid core was defined as lipid content of >10% of the total plaque area. Collagen deposition (picrosirius red) was manually classified as absent, minor, moderate or heavy staining along the entire luminal border. The burden of macrophages and SMCs was assessed by staining with antibodies against CD68 and α-actin, respectively, and was also manually classified into absent, minor, moderate or heavy staining. In alternative semi-automated computerized analyses, numbers of macrophages and SMCs were quantified on a continuous scale. Specifically, the stainings were scored as percentage of stained area to total plaque area (AnalySiS version 3.2, Soft Imaging GmbH, Munster, Germany).[27-29] Intraplaque hemorrhage (H&E and fibrin staining) was defined as the composite of plaque bleeding at the luminal side of the plaque as a result of plaque disruption, and was classified as absent or present.

To assess the overall vulnerability features of the atherosclerotic plaque, a vulnerability index was created ranging from 0 to 5, as previously described.[32, 33] Specifically, one point was given to each plaque for the following histopathological features: a lipid core >10%, low collagen load (no/minor), high macrophage burden (moderate/heavy), low SMC burden (no/minor), and presence of intraplaque hemorrhage.

Quantification of plaque levels of MCP-1 and other cytokines

Segments adjacent to those used for histopathological analysis were used for protein isolation. In brief, plaques were manually grinded at -196°C and dissolved in Tris buffer according to an in-house protocol.[24] MCP-1 concentrations (pg/mL) were quantified as part of a multiplex assay using the Luminex® platform (Austin, TX, USA) according to the manufacturer's protocol and diagnostic laboratories' standards at the clinical laboratory of the Wilhelmina Children's Hospital (WKZ, Utrecht, the Netherlands).

We further quantified the following cytokines and growth factors using established platforms: IL2, IL4, IL5, IL6, IL8, IL9, IL10, IL12, TNF-α, and IFN-γ were measured in multiplex using the human FlowCytomix system from eBioscience (cat.nr.: BMS810FF) in pg/mL. IL13, IL21, MIF, MIP1a, RANTES, MIG, IP10, Eotaxin1, TARC, PARC, MDC, sICAM1, VEGFA, and TGFB were measured in simplex assays using FlowCytomix according to the manufacturer's protocol and diagnostic laboratories' standards at the clinical laboratory of the WKZ. Metalloproteinase (MMP) activity (MMP-2, MMP-8, MMP-9) was assessed with specific Biotrak activity assays (MMP-2 RPN-2631, MMP-8 RPN-2635, and MMP-9 RPN-2634; GE Healthcare LifeSciences, Buckinghamshire, UK). Matrix metalloproteinase levels were corrected for the total protein amount and were expressed as arbitrary units. Given the different platforms used, all protein measurements were inverse-rank transformed to approach normal distributions and to ensure homogeneity in units (per 1-standard deviation [SD]).

Quantification of plasma levels of MCP-1

In a sub-sample of 374 individuals, MCP-1 levels were also quantified in the plasma using the OLINK proximity extension assays (OLINK® Bioscience) in order to explore correlations with MCP-1 plaque concentrations.

Circulating monocyte counts

In a subset of 175 patients, we measured circulating monocyte counts, as previously described.[29] In brief, a complete circulating cell profile was determined by a general

hematology cell counter (Cell Dyn 1800 Abbott, Minesota, USA). Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll gradient fractionation and stored at liquid nitrogen. After storage, PBMCs were washed and single cell suspensions were obtained through filtering over a 40 μm cell strainer (542,040 Greiner bio-one). After incubation with fluorescent antibodies, controlling for isotypes, and after exclusion of dead cells, we selected CD11B+CD115+ monocytes using the Kaluza 1.3 gating software. We derived total monocytes counts, as well as identified classical (CD14+CD16−), intermediate (CD14+CD16+) and non-classical (CD14−CD16+) monocytes.

Single-cell RNA-sequencing

In a small sub-sample of 37 individuals we applied single cell-transcriptomics (scRNAseq) to explore the source of MCP-1 levels in the atherosclerotic plaques. Methodological details are described elsewhere.[34] In brief, parts of the plaque were minced and enzymatically digested in RPMI 1640 containing 2.5 mg/mL Collagenase IV (ThermoFisher Scientific), 0.25 mg/mL DNAse I (Sigma), 2.5 mg/mL Human Albumin Fraction V (MP Biomedicals) and 1 mM Flavopiridol (Selleckchem) at 37°C for 30 minutes. Following filtration through a 70 μm cell strainer and washing, cells were suspended in RPMI 1640 with 1% Fetal Calf Serum, stained with Calcein AM and Hoechst and viable cells were sorted using Beckman Coulter MoFlo Astrios EQ. We used Mosquito® HTS (TTP Labtech) 384 wells plates, filled with 50nL lysis buffer containing CELseq2-primers, spike-ins and dinucleotide triphosphates (dNTPs) and overlaid with mineral oil to prevent evaporation. For downstream analyses we selected cells expressing between 500 and 10.000 genes and genes expressed in at least 3 cells to omit doublets and low-quality cells.[34] Data were analyzed using Seurat (Seurat_2.3.4) and log-normalized and scaled with the exclusion of unique molecular identifiers. Subsequently, canonical correlation analysis reduction was performed to identify clusters and to perform t-distributed stochastic neighbor embedding. Cell types were assigned to cell clusters by evaluating gene expression of individual cell clusters using differential gene expression and analysis with SingleR[35] against BLUEPRINT reference data.[36] Sub-clustering of identified cell

clusters was performed using canonical correlation analysis with a resolution of 0.9 or 1.5 for 15 dimensions.

Symptomatic vs. asymptomatic plaque

Patients were classified as either having an asymptomatic or symptomatic carotid plaque prior to surgery, based on their answers to a structured questionnaire regarding prior medical history and a detailed review of their medical records. Patients were considered to be symptomatic if they had suffered an acute cerebrovascular event ipsilateral to the plaque within the last 6 months. Cerebrovascular events included a supratentorial ischemic stroke, a transient ischemic attack that could be attributed to ischemia in the distribution of the respective artery, an amaurosis fugax, or a central retinal artery occlusion.

Follow-up analysis for major adverse vascular events

Patients were followed up to 3 years after surgery for potential new vascular events. The composite endpoint of any major adverse vascular event included non-fatal stroke (ischemic or hemorrhagic), non-fatal myocardial infarction, ruptured aortic aneurysm, and vascular death, defined as death of presumed vascular origin (fatal stroke, fatal myocardial infarction, sudden death, fatal aortic aneurysm rupture, fatal heart failure, other vascular death). Additional endpoints included stroke (fatal or non-fatal), acute coronary events (fatal or non-fatal myocardial infarction, unstable angina, coronary bypass or percutaneous coronary intervention, and sudden cardiac death), as well as vascular death. Outcomes occurring within the first 30 days after surgery were considered periprocedural events.[27, 37, 38] All participants underwent clinical follow-up, as detailed elsewhere.[27] Clinical endpoints were independently assessed by two clinicians at 1, 2, and 3 years after surgery through patient questionnaires, review of medical records and contact with general practitioners.

Statistical analysis

Univariable associations between inverse-rank transformed levels of MCP-1 in the plaque with other group variables were explored using Mann-Whitney U-test when comparing two groups and the Kruskal-Wallis test for three or more groups. We explored correlations between plasma and plaque levels of MCP-1 by estimating the Spearman's rho (*R*). Multivariable models were used to explore associations of plaque levels of MCP-1 (1-SD increment) with (i) plaque protein levels of a panel of 24 cytokines and growth factors (thereafter called cytokines for simplicity) and activity of three metalloproteinases, (ii) histopathological plaque vulnerability phenotypes, (iii) presence of a symptomatic vs. asymptomatic plaque, and (iv) incident major adverse vascular events. Specifically, we performed multivariable logistic regression analyses for symptomatic vs. asymptomatic plaque and for binary histopathological plaque vulnerability traits, as well as linear regression analyses for plaque cytokines and for continuous histopathological plaque vulnerability traits. For the composite vulnerability index (range 0-5), we used ordinal regression analyses. For the prospective analyses for time to new major adverse vascular events, we applied Cox proportional hazard models. Model 1 adjusted for age and sex, whereas Model 2 additionally adjusted for hypertension (self-reported history or antihypertensive medication use), diabetes (defined as self-reported history or glucose-lowering medication use), smoking status (never, former, current smoker), LDL-C levels at time of operation, use of statins or other lipid-lowering drugs, use of antiplatelet agents, estimated glomerular filtration rate (eGFR),[39] body mass index (BMI), history of cardiovascular disease (coronary artery disease, stroke, peripheral artery disease), and grade of stenosis (according to NASCET: <70%, 70-90%, 90-99%, complete occlusion). All analyses were corrected for multiple comparisons using the false discovery rate (FDR) approach. Statistical significance threshold was set at a two-sided FDR-adjusted p-value<0.05 across all analyses. Analyses were performed using R (v3.6.3; The R Foundation for Statistical Computing).

Results

A total of 1,199 patients from the Athero-Express Biobank, who had undergone carotid endarterectomy and had available MCP-1 levels in carotid plaques, were included in the current analysis (mean age 68.6±9.1 years, 36.3% females) (**Figure 1B**, **Supplemental Table I)**. MCP-1 levels in carotid plaques were higher among men compared to women, while there was no association with age or vascular risk factors including blood pressure levels, LDL-cholesterol levels, diabetes, smoking, BMI, and kidney function (**Supplemental Figure I**). There was no correlation between plaque and plasma MCP-1 levels in a sub-sample of 374 individuals with both measures available (Spearman's R=0.016, p=0.760, **Supplemental Figure II**), thus indicating that plaque MCP-1 may not be a marker of circulating MCP-1 levels. In order to detect the source of MCP-1 levels in the plaque, we applied single-cell transcriptomics in a small sample of 37 individuals and found classical CD14+/CD68+ M1 macrophages and to a lesser extent KIT+ mast cells and ACTA2+ smooth muscle cells to be the primary source of expression of *CCL2* in human atherosclerotic plaques (**Supplemental Figure III**).

Plaque MCP-1 is associated with histopathological features of plaque vulnerability

First, we explored associations of MCP-1 levels in the plaque with histopathological features of plaque vulnerability (**Figure 2**). MCP-1 levels were significantly associated with all five hallmarks of plaque vulnerability: a large lipid core (>10%), lower collagen content (no/minor), higher macrophage burden (moderate/heavy), lower SMC burden (no/minor), and presence of intraplaque hemorrhage (**Figure 2A-2B; Supplemental Table II**). Similar associations were obtained when considering macrophage and SMC burden as continuous traits (**Supplemental Table II**).

When combining the five hallmark features of plaque vulnerability traits in a validated aggregate vulnerability index,[32, 33] plaque MCP-1 levels were gradually higher among individuals with a higher score (ranging from 0-5, $p=6.3x10^{-17}$, **Figure 2C**). In models adjusting

for age and sex (model 1), as well as age, sex, and vascular risk factors (model 2), plaque MCP-1 levels were strongly and independently associated with a higher vulnerability index (Model 2: beta 0.42, 95%CI: 0.30-0.53, $p=5.4x10^{-13}$, **Figure 2D**).

Plaque MCP-1 is associated with markers of plaque inflammation and matrix turnover

We then examined whether plaque MCP-1 is associated with markers of plaque inflammation and matrix turnover. Thus, we examined the age- and sex-adjusted associations of plaque MCP-1 levels with multiple cytokines and with metalloproteinase activity in carotid plaques (**Figure 3**). We found significant associations between MCP-1 plaque levels and several cytokines involved in inflammatory cell recruitment. Specifically, we found associations with higher levels of the chemokines IL-8, PARC, TARC, and RANTES, as well with ICAM-1, an adhesion molecule involved in trans-endothelial leukocyte migration[40, 41] (all FDR-adjusted p-value <0.05 to account for multiple comparisons). Higher MCP-1 levels were further associated with higher levels of VEGF-A, a key driver of plaque neovascularization,[42] and with higher activity of the matrix metalloproteinases MMP-8, and MMP-9.[43] Similar results were obtained when further adjusting for vascular risk factors, although associations with PARC did not remain statistically significant (Model 2, **Supplemental Table III**). Finally, given the role of MCP-1 in monocyte mobilization from the bone marrow beyond monocyte chemotaxis to the plaque, we also explored correlations between MCP-1 levels in the plaque and circulating monocytes and monocyte subtypes in an overlapping sample of 179 individuals. We found no correlation with either total monocyte count or the counts of classical, intermediate, and non-classical monocytes (**Supplemental Figure IV**), thus indicating that MCP-1 plaque levels may not be a marker of monocyte egress from the bone marrow.

Plaque MCP-1 is associated with symptomatic plaques

We further explored associations between plaque MCP-1 levels and clinical plaque instability. MCP-1 levels in the plaque were higher among individuals with a symptomatic plaque (that had caused an acute cerebrovascular event) compared to individuals with asymptomatic

plaques (p=0.0001, **Figure 4A**). Following adjustments for age and sex, one SD increment in MCP-1 levels in the plaque was associated with higher odds for a symptomatic vs. asymptomatic plaque (OR: 1.31, 95%CI: 1.07-1.60, p=0.008). These associations remained significant in a model adjusting for vascular risk factors (OR: 1.36, 95%CI: 1.09-1.69, p=0.006, **Figure 4B**).

Plaque MCP-1 is associated with periprocedural stroke during carotid endarterectomy

As a last step, we explored associations between MCP-1 levels in the plaque with vascular events after carotid endarterectomy. Specifically, we looked at events occurring within the first 30 days after surgery mostly reflective of periprocedural complications, and at events occurring up to 3 years after surgery (mean follow-up 2.3 years). Plaque MCP-1 levels (1-SD increment) were associated with a higher risk of major adverse vascular events within 30-days after surgery (HR: 2.94, 95%CI: 1.32-6.50, p=0.007, **Figure 4C**) independently of age, sex, and vascular risk factors (**Supplemental Table IV**) whereas there was no such association across the entire 3-year period (HR: 1.14, 95%CI: 0.77-1.68, p=0.519, **Figure 4D, Supplemental Table IV**). MCP-1 plaque levels were further associated with a higher 30-day risk of stroke (HR: 2.32, 95%CI: 1.00-5.36, p=0.049, **Figure 4C**).

Discussion

The present study of 1,199 patients undergoing carotid endarterectomy demonstrated strong associations between plaque MCP-1 levels with multiple features of plaque vulnerability. We found associations between plaque MCP-1 levels and histopathological hallmarks of plaque vulnerability (larger lipid core, low collagen content, high macrophage burden, low SMC burden, intraplaque hemorrhage). We further found higher plaque MCP-1 levels to be associated with higher levels of inflammatory cytokines and higher activity of metalloproteinases within plaques. MCP-1 levels within plaques were higher among individuals with symptomatic plaques, as compared to asymptomatic plaques. Moreover, plaque MCP-1 levels were associated with adverse peri-procedural vascular events (time frame: 30 days after plaque removal) independently of traditional vascular risk factors. Collectively, these findings emphasize the importance of plaque MCP-1 levels in plaque vulnerability in human atherosclerosis.

While the role of MCP-1 in early stages of atherogenesis through monocyte recruitment in the plaque has been well-established,[11-13] its role in more advanced stages of atherosclerosis remained unknown. The associations reported here between MCP-1 levels in the plaque with all five hallmarks of vulnerable plaques, as well as with a composite vulnerability index, suggest that MCP-1 might be involved in mechanisms related to plaque instability in patients. Our finding of significant associations between plaque MCP-1 levels and symptomatic plaques further support this notion. Importantly, these associations were independent of conventional vascular risk factors, thus suggesting that MCP-1 signaling might contribute to plaque instability on top of established targets for secondary prevention such as LDL-cholesterol, blood pressure, and diabetes.

Our current findings on MCP-1 levels in carotid endarterectomy samples complement our recent work showing that circulating MCP-1 levels associate with ischemic stroke, coronary artery disease, and vascular death.[21-23] Whether MCP-1 levels within atherosclerotic plaques are reflective of MCP-1 activity and active monocyte recruitment remains unknown. Yet, our

finding of an association between MCP-1 levels in the plaque with multiple other cell-recruiting chemokines with a proven role in atherosclerosis,[44-47] as well as with macrophage burden in the plaque strongly suggest an association with inflammatory cell recruitment.

While MCP-1 levels within plaques were not associated with future vascular events up to 3 years after plaque removal, we found significant associations with periprocedural events, mainly stroke, occurring within the first 30 days after the procedure. Periprocedural strokes during carotid endarterectomy might have multiple causes, but have been shown to frequently originate from plaque thrombosis and embolization.[48] Although the mechanisms underlying the observed associations remain unknown, they might relate to the higher risk of periprocedural microembolization previously reported to be more common in patients with features of vulnerable plaques.[27, 49]

To our knowledge, there has been only one small phase II trial targeting MCP-1 signaling in the context of human atherosclerosis. Among 108 patients with vascular risk factors and high CRP levels, treatment with a single intravenous infusion of a humanized monoclonal antibody against the receptor of MCP-1 (CCR2), led to significant reductions in CRP levels.[50] Of note, the residual risk for clinical events among individuals with carotid plaques currently managed by best medical treatment (statins and anti-platelet agents) remains non-negligible.[51-53] Our current data in conjunction with recent genetic,[21] experimental,[15-20] and observational[22, 23] data on MCP-1 support moving towards clinical trials that target MCP-1 signaling in populations with established atherosclerotic disease.

Our study has limitations. First, the cross-sectional nature of most analyses precludes causal inferences. For example, the association between plaque MCP-1 levels and symptomatic plaques could relate to a secondary increase in plaque MCP-1 levels following the acute clinical event.[54] Similarly, the associations of plaque MCP-1 levels with other pro-inflammatory cytokines, growth factors and metalloproteinase activity can only be interpreted as correlations and not as providing a mechanistic explanation for the associations of MCP-1 with plaque vulnerability. A more detailed mechanistic exploration of the findings would require

experimental studies. Second, plaque MCP-1 levels were available only in around half of the Athero-Express population, thus potentially leading to selection bias. Yet, the demographic characteristics of our study sample matched those of the entire Athero-Express sample (**Supplemental Table I**). Similarly, there might be selection bias due to the underrepresentation of patients with asymptomatic plaques, as they are less likely to undergo carotid endarterectomy. Third, our longitudinal analyses are limited by power due to the low number of new events in the current cohort. Fourth, the pathology and mechanisms of plaque vulnerability might differ between vascular beds and these results from carotid plaques might not be extrapolated to other atherosclerotic lesions. Interestingly, previous studies have also shown involvement of MCP-1 in other vascular diseases, such as venous thromboembolism which however could not be explored in the context of the current study.

In conclusion, our study shows that among individuals undergoing carotid endarterectomy, plaque MCP-1 levels are associated with histopathological hallmarks of plaque vulnerability, a pro-inflammatory plaque profile, plaque matrix turnover, clinical plaque instability, and higher risk of periprocedural events. As such, our findings provide evidence for an involvement of MCP-1 in carotid plaque instability and further complement previous evidence from epidemiological, genetic, and experimental studies supporting the translational perspective of targeting the MCP-1/CCR2 axis in atherosclerosis.

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manuscript for intellectual content. DPVdK performed the measurements of protein levels and metalloproteinase activity in Athero-Express and revised the manuscript for intellectual content. GJdB designed Athero-Express, recruited patients for the study and revised the manuscript for intellectual content. GP designed Athero-Express and revised the manuscript for intellectual content. MD designed the current study and wrote the first draft of the manuscript.

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Highlights

- Robust evidence from epidemiological, genetic, and preclinical studies supports a role of MCP-1 in atherosclerosis. Still, the role of MCP-1 in human plaque vulnerability is unknown
- In 1,199 individuals undergoing endarterectomy due to carotid stenosis, MCP-1 plaque levels were associated with histopathological and molecular markers of plaque vulnerability
- MCP-1 levels were also associated with clinical plaque instability, as indicated by symptomatic plaques and a higher risk of periprocedural major adverse vascular events and strokes
- Our findings provide support for a role of MCP-1 in plaque vulnerability in humans

Figure legends

Figure 1. Study design. (**A**) Graphical illustration of the study design. (**B**) Flowchart detailing the number of individuals included in the respective analyses. Also shown are the number of individuals excluded from the current analyses and the reasons for exclusion.

Figure 2. Plaque MCP-1 levels associate with histopathological hallmarks of plaque vulnerability. (**A**) Graphical depiction of histopathological features of a vulnerable plaque. (**B**) Associations of plaque MCP-1 levels (1-SD increment) with the individual vulnerability features (binary traits) as derived from logistic regression analyses (adjusted for age, sex, hypertension, diabetes, current smoking, LDL-C levels at time of operation, use of lipid-lowering agents, use of antiplatelet agents, estimated glomerular filtration rate, body mass index, history of cardiovascular disease, and grade of stenosis). Shown are Odds Ratios (OR) and error bars correspond to their 95% confidence intervals (CI). (**C**) Plaque MCP-1 levels (inverse-rank transformed) in study participants across plaque vulnerability index scores (p-value derived from Kruskal-Wallis test). Shown are the median values (central line), the upper and lower quartiles (box limits) and the 1.5x interquartile range (whiskers). (**D**) Multivariable associations between MCP-1 levels in plaque (1 SD-increment) with the composite plaque vulnerability index score, as derived from ordinal regression analyses (Model 1 adjusted for age and sex, Model 2 additionally adjusted for the abovementioned vascular risk factors). Shown are beta coefficients and the error bars correspond to their 95%CI.MCP-1 plaque levels are inverse-rank transformed in all analyses.

Figure 3. Plaque MCP-1 levels associate with markers of plaque inflammation and matrix turnover. Shown are the beta coefficients and 95% confidence intervals (95%CI) derived from linear regression models for 1-SD increment in plaque MCP-1 levels (inverse-rank transformed) adjusted for age and sex. Stars indicate statistically significant results (false discovery rate-adjusted p-value <0.05).

Figure 4. Plaque MCP-1 levels associate with symptomatic versus asymptomatic plaque and periprocedural events after plaque removal. (**A**) Plaque MCP-1 levels (inverse-rank transformed) in patients with symptomatic versus asymptomatic plaques (p-value derived from Mann-Whitney U-test). Shown are the median values (central line), the upper and lower quartiles (box limits) and the 1.5x interquartile range (whiskers) (**B**) Multivariable associations between MCP-1 plaque levels (1 SD-increment) with symptomatic plaque as derived from logistic regression analyses (vascular risk factors in the second model include hypertension, diabetes, current smoking, LDL-C levels at time of operation, use of lipid-lowering agents, use of antiplatelet agents, estimated glomerular filtration rate, body mass index, history of cardiovascular disease, and grade of stenosis). Shown are Odds Ratios (OR) and error bars correspond to their 95% confidence intervals (CI). (**C, D**) Associations of plaque MCP-1 levels (1-SD increment) with new adverse vascular events within (**C**) 30 days (periprocedural events) and (**D**) 3 years after surgery, as derived from Cox regression analyses (adjusted for age, sex, and the abovementioned vascular risk factors). Shown are Hazard Ratios (OR) derived from Cox regression analyses and error bars correspond to their 95%CI. MCP-1 plaque levels are inverse-rank transformed in all analyses.