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Mast Cell Distribution in Human Carotid Atherosclerotic Plaque Differs Significantly by Histological Segment

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WHAT THIS PAPER ADDS

Mast cells are found in atherosclerotic plaques and have various direct and indirect effects on plaque composition, progression, and destabilisation, and have been suggested as therapeutic targets for atherosclerosis. Treatment can be assessed on a histological level; however, the intersegment distribution of mast cells is unknown. This study on carotid plaques revealed that there are large intersegment differences in mast cells per mm². It is recommended that a standardised segment is used for mast cell analysis. Furthermore, it is advocated that the specification of the plaque segment be included in the reporting standard of biobank analyses.

Objective: Mast cells (MCs) are important contributors to atherosclerotic plaque progression. For prospective studies on mast cell contributions to plaque instability, the distribution of intraplaque MCs needs to be elucidated. Plaque stability is generally histologically assessed by dividing the plaque specimen into segments to be scored on an ordinal scale. However, owing to competitive use, studies may have to deviate to adjacent segments, yet intersegment differences of plaque characteristics, especially MCs, are largely unknown. Therefore, the hypothesis that there is no segment to segment difference in MC distribution between atherosclerotic plaque segments was tested, and intersegment associations between MCs and other plaque characteristics was investigated.

Methods: Twenty-six carotid atherosclerotic plaques from patients undergoing carotid endarterectomy included in the Athero-Express Biobank were analysed. The plaque was divided in 5 mm segments, differentiating between the culprit lesion (segment 0), adjacent segments (−1/+1) and more distant segments (−2/+2) for the presence of MCs. The associations between the intersegment distribution of MCs and smooth muscle cells, macrophage content, and microvessel density in the culprit lesion were studied.

Results: A statistically significant difference in MCs/mm² between the different plaque segments ($p < .001$) was found, with a median of 2.79 (interquartile range [IQR] 1.63 – 7.10) for the culprit lesion, 1.34 (IQR 0.26 – 4.45) for the adjacent segment, and 0.62 (0.14 – 2.07) for the more distant segment. Post hoc analyses showed that intersegment differences were due to differences in MCs/mm² between the culprit and adjacent segment ($p = .037$) and between the culprit lesion and the more distant segment ($p < .001$). MCs/mm² in multiple different segments were positively correlated with microvessel density and macrophage content in the culprit lesion.

Conclusion: MC numbers reveal significant intersegment differences in human carotid plaques. Future histological studies on MCs should use a standardised segment for plaque characterisation as plaque segments cannot be used interchangeably for histological MC analyses.

Keywords: Atherosclerosis, Carotid arteries, Carotid Endarterectomy, Mast cells, Plaque composition

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INTRODUCTION

Atherosclerosis is a chronic inflammatory arterial disease characterised by the accumulation of inflammatory cells, lipids, and fibrous elements causing luminal narrowing, plaque erosion, or rupture.¹ Unstable plaques that are prone to rupture are characterised by pathological characteristics such as a large lipid core, a thin fibrous cap,

intraplaque haemorrhage (IPH) and infiltrates of pro-inflammatory cells such as macrophages, T cells, and mast cells (MCs).²

In histological studies, plaque specimens are generally divided into segments and scored on an ordinal scale, which is the gold standard for histological assessment of plaque stability.^{3,4} The culprit lesion is defined as the plaque segment showing the largest plaque burden, as determined by visual assessment. The culprit lesion typically contains the most unstable plaque characteristics,^{5,6} and is therefore the segment preferred for histological analysis. Due to the increasing interest in revealing the relationship between atherosclerosis and clinical endpoints using atherosclerotic plaques, biobank studies have acquired a unique cornerstone position. As a consequence, owing to competitive use of this segment, the culprit lesion is not always available for histological assessment purposes. For plaque analysis, adjacent or more distant segments then become of interest.

MCs are pluripotent inflammatory cells of which the numbers in atherosclerotic plaques increase over the course of plaque progression.^{7,8} MCs have been implicated in pro-atherogenic mechanisms leading to plaque instability such as thinning of the fibrous cap and intraplaque neo-vascularisation.^{9,10} In addition, MC per mm² in the culprit lesion were found to be associated with the unstable plaque characteristics, IPH, and microvessel density. All three (i.e., IPH, increased microvessel density, and MCs in the culprit lesion) are independently associated with an increased risk of future cardiovascular events in the three years after carotid endarterectomy (CEA).^{8,11} Patients at higher risk of developing a new cardiovascular event during this period (i.e., 24% – 25.2% of the population undergoing CEA) are eligible for more intensive medical treatment.^{11,12} This more intensive medical treatment may include more rigorous treatment with lipid lowering drugs and it may be directed towards drugs that reduce inflammation, such as canakinumab or colchicine.^{13,14}

In vivo and *in vitro* studies have shown that MCs may also be considered as a therapeutic target for the reduction of inflammation in atherosclerosis. There is increasing evidence that statins have direct and indirect inhibitory effects on MCs via various inflammatory pathways.^{15,16} In addition, animal studies have shown that treatment with anti-IgE therapy directed against MC activation can limit the progression of atherosclerosis.¹⁷ Targeting MCs may therefore be a valid approach with the potential effectively to inhibit atherosclerosis progression and the cardiovascular consequences in humans. To validate the effect of MC targeted therapy on the atherosclerotic plaque, the natural inter-segment distribution of MCs within the atherosclerotic plaque needs to be clarified. This is not only important to be included in study protocols but should also be a part of the reporting standards in histological evaluation studies, especially in studies where histological specimens are used for outcome analysis.

Intersegment differences of multiple plaque characteristics such as IPH, calcification, macrophage, and smooth muscle cell (SMC) content has been studied before. Semi-

quantitative ratings of these plaque characteristics tend to deviate with increasing distance from the culprit lesion. Although most differences were reported to be minor, in the majority of cases this meant there was at most a difference of one category on the ordinal histological assessment scale.³ However, until now, the distribution of MCs throughout different atherosclerotic plaque segments in patients undergoing CEA has not been studied numerically. Therefore, the segment dependent distribution of plaque MCs in a random sample of patients undergoing CEA was investigated. Additionally, the association of MCs in different segments with local plaque characteristics indicative of plaque instability such as microvessel density, SMC, and macrophage content was examined.

MATERIALS AND METHODS

Patients

For this study, a subset of 26 plaques from the Athero-Express Biobank (AE) sub-study (www.atheroexpress.nl) were included. The AE is an ongoing observational prospective biobank study. A complete outline of the study design has been published previously.^{11,18} In short, all consecutive patients undergoing CEA at the University Medical Centre Utrecht and St. Antonius Hospital Nieuwegein, The Netherlands, were included. Indications for CEA were evaluated by a multidisciplinary vascular team according to European Society for Vascular Surgery guidelines.¹⁹ Patients completed standardised questionnaires at baseline to collect data regarding cardiovascular risk factors, medical history, medication use, and basic laboratory parameters. These questionnaires were verified against medical records. A pre-operative blood sample was taken, centrifuged, and subsequently stored in a –80°C freezer until further use. During CEA the plaques were carefully removed *in toto* and immediately transferred to the laboratory for further processing. Inclusion criteria for the current study were availability of all three segments: the culprit lesion (0 segment); the adjacent segment; and more distant segments, the latter two from either side of the culprit lesion (+2, +1 or –1, –2). The study was conducted in accordance with Declaration of Helsinki, and all patients provided written informed consent. Ethical approval for the study was obtained from the institutional ethics boards of both participating hospitals.

Plaque processing and semi-quantitative assessment

The sample collection protocol of the AE has been described in detail.²⁰ In summary, after excision, the total length of the atherosclerotic plaque was measured. Subsequently, the plaque was dissected by a dedicated technician into longitudinal segments that each had a length of 5 mm. The plaque segment with the largest plaque burden was defined as the culprit lesion. Adjacent segments were numbered either +1 or –1, and more distant segments either +2 or –2. The remaining segments were numbered and stored for future use. The segments were fixed in 4%

formaldehyde, decalcified for one week in ethylenediaminetetraacetic acid, and embedded in paraffin. The 5 mm segments were cut into 5 μ m slices on a microtome and used for immunohistochemical staining. Haematoxylin and eosin staining was used to obtain a general overview, including calcifications and the lipid core, picrosirius red and elastin von Gieson staining for collagen, α -actin staining for SMCs, and CD68 for macrophages. Subsequent semi-quantitative analyses of plaque characteristics were performed with 40 \times magnification on a microscope. Plaque characteristics were scored by two independent observers who were blinded to clinical data with a good intra-observer and interobserver reproducibility ($\kappa = 0.6 - 0.9$).³ The following characteristics were scored on an ordinal scale according the standardised AE protocol: fat; collagen; calcifications; and overall plaque phenotype.¹¹ IPH was scored as present or absent. Macrophages and SMC content, and intraplaque vessels (using CD34 antibodies) were quantified with analysis software (AnalySIS 3.2; Soft Imaging Systems, Munster, Germany). SMCs and macrophage content were expressed as the average percentage of positive staining of the plaque area of three representative regions of interest of the plaque selected by an experienced technician at a 40 \times magnification. CD34⁺ intraplaque vessels were counted in three hotspots with the highest vessel density and the average number per mm² was calculated, as described previously.²¹

Numerical mast cell distribution

To assess MC numbers, the plaque segments were pre-treated with a citrate buffer and then stained with a monoclonal mouse antibody against tryptase (dilution 1:400; Dako-Cytomation, Carpinteria, CA, USA). Powervision poly-horseradish peroxidase against mouse IgG (Immuno-Logic, Duiven, the Netherlands) was used as secondary antibody, after which tryptase staining was visualised with Liquid Permanent Red (Dako-Cytomation). The remaining tissue was visualised with a haematoxylin (blue) counter-stain. Thereafter, slides of the plaque segments were scanned at 4 \times magnification using slide scanners and stored as digital images. Using Olympus cellSens Dimension 1.15 software, plaque size was determined for each segment separately by outlining the total plaque area, and all MCs were counted manually. Previous research has shown that this method has good intra-observer variability (Spearman's $\rho = .947$; $p < .001$).⁸ In the histological analyses, the total number of MCs is expressed as MCs/mm² plaque size per plaque segment.

Statistical analysis

Continuous baseline characteristics were summarised as mean and standard deviation, or median and interquartile range (IQR). Categorical baseline characteristics were expressed as frequencies. MC numbers were not normally distributed; non-parametric tests were used to determine differences or correlations. The Kruskal–Wallis test was used to test for differences between the three different

plaque segments (culprit lesion, adjacent, and more distant segments). Subsequently, for the post hoc analysis the pairwise Wilcoxon rank sum test was used to compare group medians and determine the differences between two segments. The Spearman rank correlation coefficient was used to test for correlations between MC numbers and microvessel density, SMC, and macrophage content. All p values resulted from two tailed hypothesis testing. A p value $< .05$ indicated statistical significance. Analyses were performed in R statistical software version 3.6.2 (<https://www.r-project.org/>).

RESULTS

Baseline characteristics

A total of 26 plaques were included for which the patient characteristics are summarised in Table 1. The study population consisted predominantly of men (81%) with a mean age of 70 years; nearly two thirds of the patients had been symptomatic (65%). At inclusion, the majority of the study participants had hypertension (96%) and hypercholesterolaemia (81%), and used lipid lowering and anti-hypertensive drugs (81% and 77%, respectively). These baseline characteristics are in line with the baseline characteristics of the complete AE cohort.

Table 1. Baseline characteristics of patients included in this study

Patient characteristic	Patients (n = 26)
Age – y	70.4 \pm 8.9
Male	21 (81)
Body mass index – kg/m ²	25.9 \pm 3.2
Systolic blood pressure – mmHg	163.3 \pm 20.6
Diastolic blood pressure – mmHg	86.5 \pm 14.2
Current smoker	7 (27)
Hypertension	25 (96)
Hypercholesterolaemia	21 (81)
Diabetes mellitus	7 (27)
History of TIA/stroke	9 (35)
History of PAOD	4 (15)
History of peripheral intervention(s)	6 (23)
History of CAD	8 (31)
Blood pressure medication use	20 (77)
Statin use	21 (81)
Antiplatelet use	21 (81)
Oral anticoagulants	4 (15)
Total cholesterol – mmol/L	4.57 (3.6–5.2)
HDL – mmol/L	1.27 (0.95–1.61)
LDL – mmol/L	1.98 (1.58–3.23)
Triglycerides – mmol/L	1.31 (1.09–2.16)
Kidney function, eGFR – mL/min/1.73 m ²	62.7 (51.3–73.7)
Symptomatic carotid stenosis	17 (65)
Ocular	2 (8)
TIA	6 (23)
Stroke	9 (35)

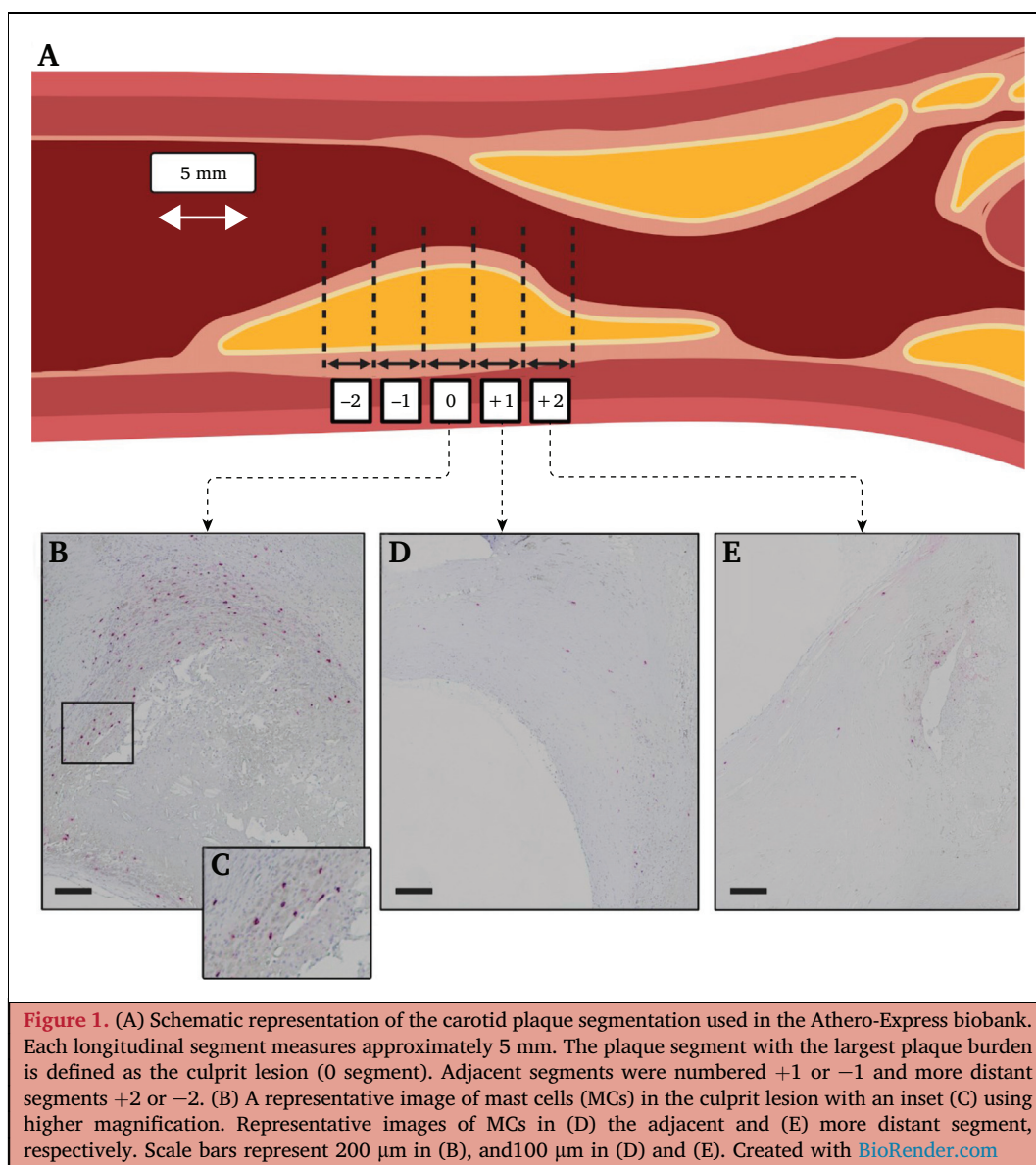
Data are given as n (%), mean \pm standard deviation, or median (interquartile range). TIA = transient ischaemic attack; PAOD = peripheral arterial occlusive disease; CAD = coronary artery disease; HDL= high density lipoprotein; LDL= low density lipoprotein; eGFR = estimated glomerular filtration rate.

Segment to segment distribution of mast cells per mm²

MCs were found to be present in the culprit lesion, and adjacent and more distant segments (Fig. 1). A statistically significant difference in MC number per mm² between the different plaque segments ($p < .001$) was observed, with a median of 2.79 MCs/mm² (IQR 1.63 – 7.10) for the culprit lesion, 1.34 MCs/mm² (IQR 0.26 – 4.45) for the adjacent segment, and 0.62 MCs/mm² (IQR 0.14 – 2.07) for the more distant segment. Post hoc analyses showed that this was due to differences in MCs/mm² between the culprit lesion and the adjacent segment ($p = .037$) and between the culprit lesion and the more distant segment ($p < .001$) (Fig. 2). In a separate subgroup analysis stratified for symptom status a statistically significant difference in MCs/mm² between the segments was found in plaques from symptomatic patients ($p = .003$) but not in plaques from asymptomatic patients ($p = .26$) (Fig. 3). Post hoc analyses revealed that the difference in MCs/mm² between plaque

segments of symptomatic patients was a result of the difference in MCs/mm² between the culprit lesion and the more distant segment ($p < .001$) and between the adjacent segment and the more distant segment ($p = .049$). Overall, the MCs/mm² per segment type between symptomatic and asymptomatic patients were not statistically different (Table 2).

MCs/mm² in the culprit lesion were positively correlated with MCs/mm² in the adjacent segments ($r = .44$, $p = .027$) and with MCs/mm² in the more distant segments ($r = .43$, $p = .031$) (Table 3). In addition, MCs/mm² in the adjacent segments were positively correlated with MCs/mm² in the more distant segments ($r = .49$, $p = .013$). In a subgroup analysis stratified for symptom status, MCs/mm² in the culprit lesion were not significantly correlated with the MCs/mm² in the adjacent segments, or with MCs/mm² in the more distant segments in plaques from asymptomatic and symptomatic patients. A statistically significant positive



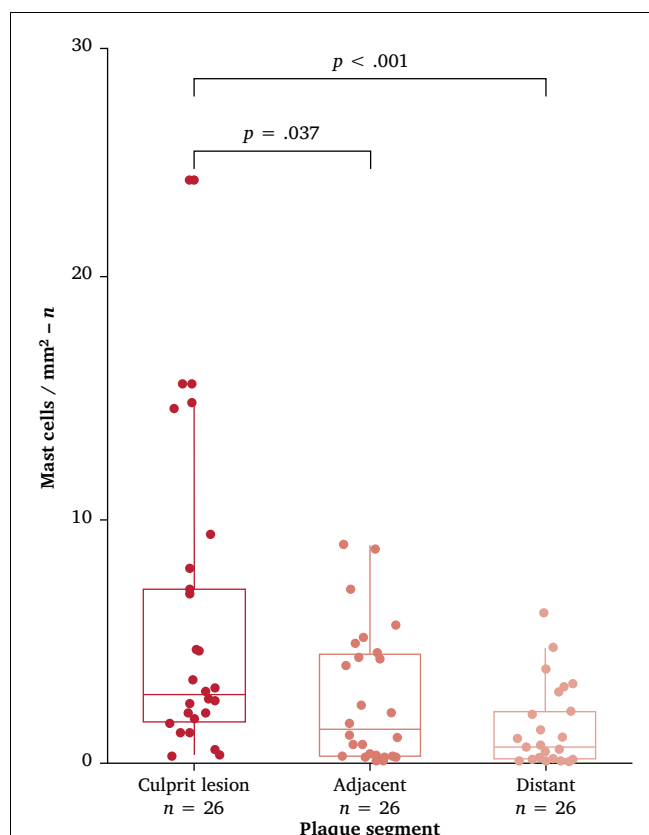


Figure 2. Segment to segment distribution of mast cells (MCs)/mm² of atherosclerotic plaque from the carotid artery. The difference was statistically significant between plaque segments ($p < .001$) owing to differences in MCs/mm² between the culprit lesion and the adjacent segment ($p = .037$) or distant segment ($p < .001$).

correlation was found between MCs/mm² in the adjacent segment with MCs/mm² in the more distant segment in plaques from symptomatic patients ($r = .67$, $p = .004$) but not in plaques from asymptomatic patients (Table 4).

Mast cells per mm² and plaque characteristics

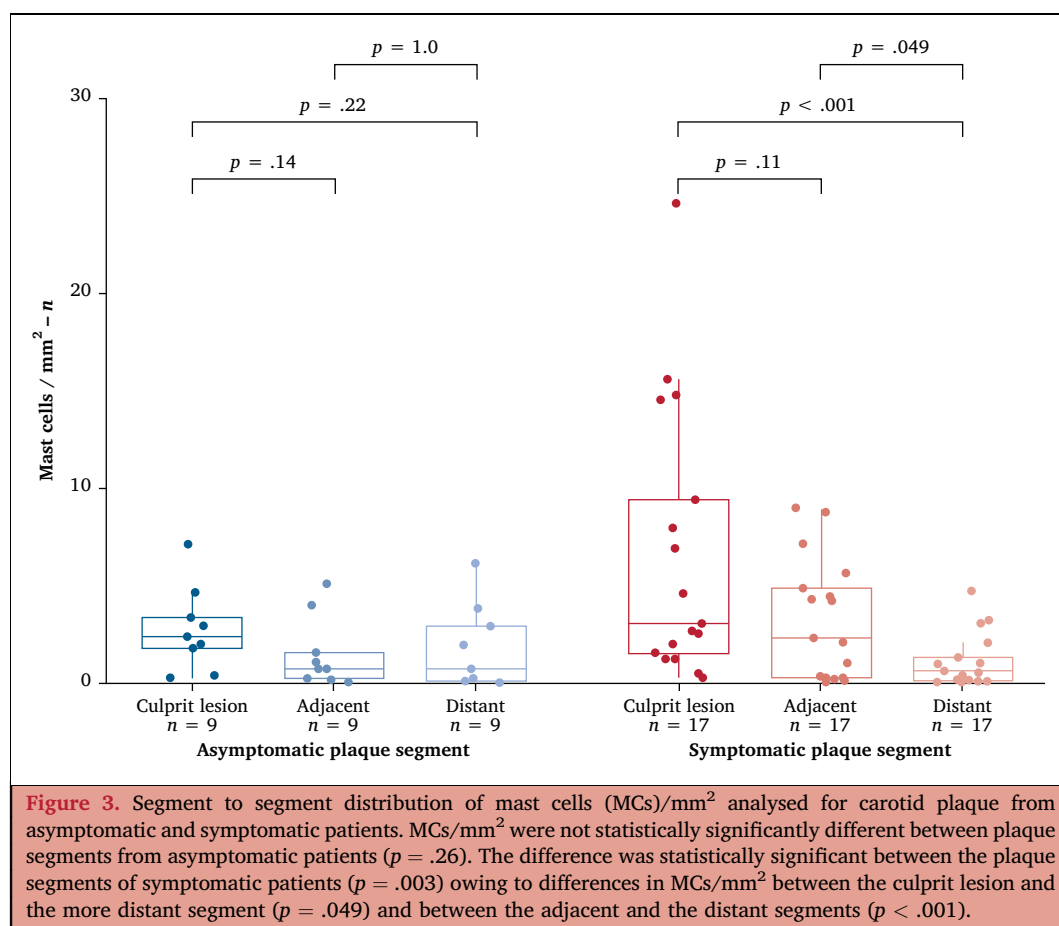
A positive correlation between the MCs/mm² in the culprit lesion and MCs/mm² in the adjacent segment with microvessel density in the culprit lesion was observed ($r = .44$, $p = .024$ and $r = .39$, $p = .048$, respectively) (Table 3). MCs/mm² in the more distant segments were not correlated with microvessel density in the culprit lesion. Furthermore, the macrophage content of the culprit lesion was positively associated with MCs/mm² in the culprit lesion ($r = .53$, $p = .005$), adjacent ($r = .40$, $p = .041$), and distant segments ($r = .58$, $p = .002$). No correlation was observed between the SMC content in the culprit lesion and the MCs/mm² in the culprit lesion, and adjacent and more distant segments. In a subgroup analysis including only plaques from symptomatic patients, a statistically significant correlation was found between microvessel density and macrophages in the culprit lesion with MCs/mm² in the adjacent segment ($r = .62$, $p = .008$ and $r = .50$, $p = .045$, respectively). In asymptomatic plaques, a statistically

significant positive correlation was found between microvessel density in the culprit lesion and MCs/mm² in the culprit lesion ($r = .95$, $p < .001$). In addition, MCs/mm² in the culprit lesion were positively correlated with macrophages in the culprit lesion ($r = .73$, $p = .031$).

DISCUSSION

In this study, the segment to segment distribution of MC numbers in the culprit lesion, and the adjacent and more distant segments of carotid atherosclerotic plaques was investigated. The highest MC/mm² were found in the culprit lesion and this score was significantly different from the MC/mm² in the adjacent and the more distant segment. However, MC/mm² between the adjacent segment vs. the more distant segment did not differ significantly. This observation was partly seen in plaques from symptomatic patients as shown in a separate subgroup analysis and not for asymptomatic plaques. The highest MC numbers per mm² were observed in the symptomatic culprit lesion and the lowest in the symptomatic more distant segment. This may indicate that MCs are upregulated in the segment that generally contains the most unstable plaque characteristics.^{5,6} A possible explanation might be that during plaque progression the number of MCs and other inflammatory cells increases as a result of an increase in extravasation of inflammatory cells into the plaque via newly formed microvessels. Moreover, MCs have been shown to co-localise with plaque microvessels, and their numbers increase in the areas where plaque microvessels are abundantly present. In the current study, MCs/mm² in the culprit lesion and in the adjacent segment were both correlated with microvessel density, which is consistent with earlier findings.⁸ The correlation does not say anything about cause and effect. It might be possible that MCs are responsible for the induction of new vessels via the secretion of basic fibroblast growth factor and vascular endothelial growth factor, or that MCs enter the plaque via these microvessels after their formation.^{10,22} Acute MC activation in more advanced and unstable plaque can result in leakiness of these microvessels, which may lead to IPH.²³

When activated directly MCs induce leucocyte recruitment and enhance adhesion molecule expression on endothelial cells. By doing so, MCs affect plaque stability.²⁴ This underlying role of MCs might be an explanation for the observed positive significant correlation between macrophages and MCs in all three segments. CD68⁺ macrophages were found to be abundantly present in the shoulder region and adventitia of atherosclerotic plaque, two sites that are also enriched with MCs.^{25,26} MCs have various additional effects on macrophages in the atherosclerotic plaque. Activation of MCs contributes to the conversion of macrophages into foam cells.²⁷ MCs can proteolyse high density lipoprotein and thereby prevent the high affinity efflux of cholesterol out of foam cells.²⁸ MCs can also induce apoptosis of macrophages, which contributes to a larger necrotic core,²⁴ and are thus also associated with plaque instability. A limitation of the current study was that



evaluation of a correlation between MC distribution in the plaque with, for example, macrophages was not possible owing to the unavailability of the differential white blood cell counts for the included patients.

Taken together, these associative data suggest that MCs play an active key role in the underlying processes that contribute to plaque neovascularisation and vulnerability. Therefore, MCs may be an important therapeutic target to inhibit the progression of atherosclerosis, especially in patients who remain at residual risk of a future cardiovascular event. A 2019 study found that the major pathway for the activation of MCs in atherosclerotic plaque was mediated by IgE and that MCs were highly activated.²⁹ In a murine study, treatment with an anti-IgE neutralising antibody reversed vascular inflammation and accelerated

atherosclerotic lesion formation in cholesterol fed *Ldlr*^{-/-} *slgM*^{-/-} mice.¹⁷ These data suggest that anti-IgE treatment may be beneficial in inhibiting atherosclerosis.³⁰ To test this hypothesis, a prospective study (<https://www.trialregister.nl/trial/8294>) will be launched to evaluate the therapeutic effect of anti-IgE treatment on MCs and plaque composition in patients undergoing CEA. The results of the current study will be directly incorporated into the design of the prospective study.

In addition, there is growing evidence that statins have inhibitory effects on MCs and therefore limit the development and progression of atherosclerosis.^{31,32} In this study, the relationship between MCs/mm² and statin intake could not be analysed owing to limited power. A correlation analysis against the lipid profile before CEA, corrected for statin intake, revealed no significant correlations. In previous AE biobank work, the association between statin intake and MCs/mm² was analysed and no statistically significant association was found.⁸

This is the first study to report numerical data on the distribution of MC numbers of different segments of carotid atherosclerotic plaques. One study described MC distribution in carotid plaque specimens; however, numerical data on the MC distribution were not reported.⁷ In addition, the intersegment differences of MCs were not assessed. Intersegment differences in multiple plaque characteristics such as IPH, calcification, macrophages, and SMC content have

Table 2. Differences in mast cells (MCs)/mm² between plaque segments of atherosclerotic carotid plaques from asymptomatic and symptomatic patients

MCs/mm ² of plaque segment	Asymptomatic (n = 9)	Symptomatic (n = 17)	p value
Culprit lesion	2.38 (1.79–3.37)	3.06 (1.58, 9.39)	.27
Adjacent segment	0.73 (0.26–1.57)	2.35 (0.28–4.86)	.25
Distant segment	0.73 (0.13–2.91)	0.62 (0.15–1.33)	.73

Data are presented as median (interquartile range).

Table 3. Correlation between mast cells (MCs)/mm² across segments with microvessel density, macrophages, and smooth muscle cells (SMCs) in the culprit lesion of 26 symptomatic or asymptomatic carotid plaques

Plaque characteristic	MCs/mm ² in culprit lesion		MCs/mm ² in adjacent segment		MCs/mm ² in distant segment	
	<i>r</i>	<i>p</i> value	<i>r</i>	<i>p</i> value	<i>r</i>	<i>p</i> value
MCs/mm ² in culprit lesion			.44	.027	.43	.031
MCs/mm ² in adjacent segment	.44	.027			.49	.013
MCs/mm ² in distant segment	.43	.031	.49	.013		
Microvessel density in culprit lesion	.44	.024	.39	.048	.34	.088
Macrophages in culprit lesion	.53	.005	.40	.041	.58	.002
SMCs in culprit lesion	.15	.46	.09	.65	-.22	.27

Data are presented as Spearman's rank correlation coefficient (*r*).

Table 4. Correlation between mast cells (MCs)/mm² across segments with microvessel density, macrophages, and smooth muscle cells (SMCs) in the culprit lesion of nine asymptomatic and 17 symptomatic carotid plaques

Plaque characteristic	MCs/mm ² culprit lesion		MCs/mm ² adjacent segment		MCs/mm ² distant segment	
	<i>r</i>	<i>p</i> value	<i>r</i>	<i>p</i> value	<i>r</i>	<i>p</i> value
<i>Symptomatic plaque</i>						
MCs/mm ² in culprit lesion			.44	.076	.47	.058
MCs/mm ² in adjacent segment	.44	.076			.67	.004
MCs/mm ² in distant segment	.47	.058	.67	.004		
Microvessel density in culprit lesion	.24	.35	.62	.008	.47	.056
Macrophages in culprit lesion	.47	.062	.50	.045	.40	.12
Smooth muscle cells in culprit lesion	.28	.28	.02	.94	-.34	.18
<i>Asymptomatic plaque</i>						
MCs/mm ² in culprit lesion			-.02	.98	.32	.41
MCs/mm ² in adjacent segment	-.02	.98			.18	.64
MCs/mm ² in distant segment	.32	.41	.18	.64		
Microvessel density in culprit lesion	.95	<.001	-.10	.80	.13	.75
Macrophages in culprit lesion	.73	.031	.37	.34	.77	.021
SMCs in culprit lesion	.58	.22	.23	.55	-.13	.74

Data are presented as Spearman's rank correlation coefficient (*r*). MC = mast cell.

been published,³ suggesting that plaque morphology can differ between plaque segments. Macrophages and SMC content showed the lowest variability between segments, while other plaque characteristics such as IPH and calcification had a high variability between segments. However, the variability in macrophages and SMCs increased when the distance between plaque segments increased,³ which is similar to the observed MC distribution between segments in this study.

In conclusion, this study revealed large intersegment differences in MC numbers, especially between the culprit lesion and the adjacent segment, and between the culprit lesion and the more distant segment. When comparing the adjacent segment with the more distant segment the intersegment differences were not significantly different. Although limited in sample size, these results highlight the need for standardisation in future studies regarding the use of plaque specimens for the histological assessment of MCs. Based on the findings, it is recommended that one standard segment is used for MC plaque analysis for future histological studies, as plaque segments are not interchangeable when it comes to histological analysis for MC distribution. It

is recommended that the culprit lesion segment is used when available. If the culprit lesion is unavailable, the adjacent segment can be used as a proxy in symptomatic individuals.

CONFLICTS OF INTEREST

None.

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