



Macrophage Subtypes in Symptomatic Carotid Artery and Femoral Artery Plaques

S. Shaikh^a, J. Brittenden^a, R. Lahiri^b, P.A.J. Brown^b, F. Thies^a, H.M. Wilson^{a,*}

^a Division of Applied Medicine, University of Aberdeen, Foresterhill, Aberdeen AB25 2ZD, UK

^b Department of Pathology, Aberdeen Royal Infirmary, Aberdeen, UK

WHAT THIS PAPER ADDS?

- Atherosclerotic plaques from distinct anatomical locations exhibit differences in their cellular and biochemical composition and importantly the proportion of inflammatory macrophage subsets.
- Compared to femoral plaques from patients with chronic limb ischemia, symptomatic carotid lesions exhibit increased lipid and inflammatory cell infiltration, a greater proportion of M1-like pro-inflammatory macrophages, and a decreased proportion of atheroprotective M2-cells.
- Identification and evaluation of more specific drug therapies which target M1-type macrophages in symptomatic carotid disease could prove to be a valuable therapeutic intervention.

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ABSTRACT

Objective: To compare differences in macrophage heterogeneity and morphological composition between atherosclerotic plaques obtained from recently symptomatic patients with carotid artery disease and femoral plaques from patients with severe limb ischemia.

Design: Experimental study.

Methods: Plaques were obtained from 32 patients undergoing carotid endarterectomy and 25 patients undergoing common femoral endarterectomy or lower limb bypass. Macrophages and T cell numbers were detected in plaque sections by immunohistochemistry and anti CD68 and CD3 antibodies. Dual staining for CD68 and M1- and M2-macrophage markers and morphometric analysis of hematoxylin and eosin stained plaque sections was performed.

Results: Carotid plaques had significantly increased percentage areas of confluent lipid and leukocytic infiltrates. In contrast, areas of fibroconnective tissue were significantly greater in femoral plaques and percentage areas of confluent calcification and collagen were elevated. Carotid artery plaques had greater numbers per plaque area of macrophages and T cells consistent with a more inflammatory phenotype. Proportions displaying M1-activation markers were significantly increased in the carotid compared to femoral plaques whereas femoral plaques displayed a greater proportion of M2-macrophages.

Conclusion: Plaques from patients with recently symptomatic carotid disease have a predominance of M1-macrophages and higher lipid content than femoral plaques, consistent with a more unstable plaque.

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Introduction

Atherosclerosis is a systemic inflammatory disease characterized by the formation of atheromatous plaques within arteries.¹ These plaques can be classified as rupture prone and unstable as characterized by a necrotic lipid core, increased inflammatory cell

infiltrate and reduced fibrous cap thickness, or stable and fibrous with little lipid and inflammatory infiltrate and increased cap thickness.² The presence of inflammatory cells and notably macrophages within plaques has long been associated with plaque destabilization, rupture and thrombosis.³ Recent interest has focussed on the heterogeneous role of inflammatory macrophages in the initiation, progression and vulnerability of atherosclerotic lesions.^{3–6} Macrophage heterogeneity and broad ranging functions are dictated by microenvironmental stimuli.^{4,7} Classically activated or M1-macrophages have anti-microbial and tissue-destructive

* Corresponding author. Tel.: +44 1224 437350; fax: +44 1224 437348.

E-mail address: h.m.wilson@abdn.ac.uk (H.M. Wilson).

properties. They secrete pro-inflammatory cytokines, chemokines and reactive oxygen and nitrogen species, metalloproteinases and tissue factor that are associated with plaque destabilization, rupture and thrombosis.^{4–7} Alternatively activated or M2-macrophages are anti-inflammatory, tissue-reparative, immunosuppressive and reduce inflammation by clearing apoptotic cells and tissue debris. In established plaques, it is proposed these would principally be anti-atherogenic and enhance plaque stability.^{4–8} In atherosclerosis, other characteristic subsets e.g. M_{0x} macrophages are becoming recognized⁹ and the contribution of macrophages to plaque stability and clinical outcome will be influenced by the balance of the distinct subtypes present.^{4–6}

Plaques within the internal carotid artery territory often thrombo-embolise, resulting in stroke, transient ischemic attacks and amaurosis fugax.² Plaques within femoral arteries often give rise to flow-limiting symptoms such as intermittent claudication, rest pain, ulceration and gangrene. It might be expected that plaques which had been associated with a recent thrombo-embolic event would exhibit greater biochemical and compositional features of instability and more inflammatory, destabilizing macrophages, as compared to plaques which were associated predominantly with stable flow-limiting symptoms, although evidence in the literature to support this contention is scanty and even conflicting.^{10–12}

Recognizing the types and roles of inflammatory macrophages within atheromatous plaques and how they relate to plaque composition or stability is important to understand the pathogenesis of lesions and may have significant implications for both imaging vulnerable plaques and tailoring appropriate therapy. This study was designed to fully evaluate the morphological composition of atherosclerotic plaques from recently symptomatic patients with carotid disease and femoral plaques from patients with severe limb ischemia, focusing on the differences in subsets of inflammatory macrophages.

Materials and Methods

Patients

Thirty two consecutive patients presenting with symptomatic, significant carotid stenosis (>50% stenosis according to the NASCET criteria)¹³ and 25 patients presenting with severe limb ischemia and significant stenosis of either the common femoral arteries (23 patients) or the superficial femoral arteries (2 patients), which were due to undergo surgery, were recruited. Ethical approval was obtained from the North East of Scotland Ethical Committee. Informed written consent was obtained from all patients. All patients underwent secondary prevention therapy according to National guidelines (Scottish Intercollegiate Guidelines network) for the treatment of symptomatic cerebrovascular disease and peripheral arterial disease.^{14,15}

Plaque processing and histopathology

The excised carotid or femoral endarterectomy specimens were cut into 7 × 2–3 mm segments as shown in Fig. 1. Pre-defined segments were designated for both histology and immunohistochemistry and fixed in 5% phosphate buffered formaldehyde. After decalcification in 5% formic acid for 24 h, the sections were embedded in paraffin.

Histopathology and plaque morphometry

Hematoxylin and eosin stained sections of plaque were analyzed using the modified American Heart Association (AHA)

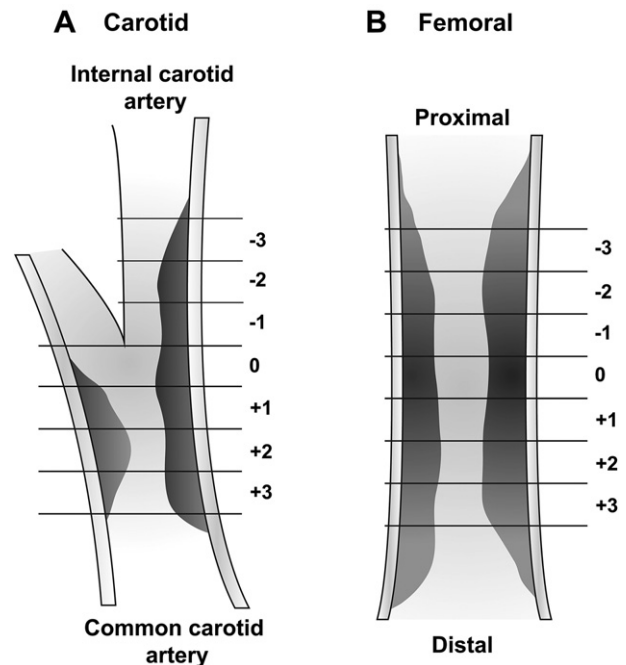


Figure 1. Schematic presentation of segmentation of the endarterectomy specimen. Plaques were cut into 7 segments of 2–3 mm in thickness depending on the size of plaque. For carotid plaques, segment 0 was taken as the last complete transection of the common carotid artery at its bifurcation. For femoral plaques segment 0 represents the area of maximum stenosis, taken as the center of the plaque. Segments –3, 0 and +2 was further sectioned for histological and immunohistochemical analysis.

classification¹⁶ by an experienced consultant pathologist (PAJB) blinded to the clinical data.

Morphometric analysis of hematoxylin and eosin stained plaque sections was based on criteria from published studies¹⁷ and performed by an experienced pathologist (RL) blinded to the clinical data. The total plaque area was measured in each plaque by captured digital images and tracing around the perimeter of confluent areas. Confluent zones greater than 0.02 mm² of (i) lipid, (ii) calcification, (iii) fibroconnective tissue in which occasional scattered lymphocytes, lipid laden macrophages or spotty calcification (defined as fibroconnective tissue) and (iv) fibroconnective tissue with obvious lymphocytic or mixed lymphocytic and macrophage cellular infiltrate, were determined. These confluent zones were classified as illustrated in Fig. 2. Measurements were expressed as mm². Collagen content was determined after Picrosirius red staining. Areas were defined as percentage areas of the total endarterectomy specimens so that relative proportions of the components in each plaque were comparable. The intra-observer variation was <3%. In all cases the median value from sections –3, 0 and +2 is reported.

Cellular composition: immunohistochemistry

Sections from the 3 areas of plaque (–3, 0 and +2) were deparaffinized and dehydrated followed by microwaving in citrate buffer (pH 6.0) for antigen retrieval and endogenous peroxidase activity quenched with 3% (vol/vol) H₂O₂ in methanol. Plaque sections were batch stained using a Dako autostainer. Macrophages and T-cells were detected by anti-CD68 (KP1) and anti-CD3 (F7.2.38) antibodies (Dako, Ely, UK) respectively, and the DAKO Envision Detection Kit K5007. Specific brown staining was developed by incubation with Chemate diaminobenzidine (Dako). Slides were counterstained with hematoxylin and nuclei blued with Scots

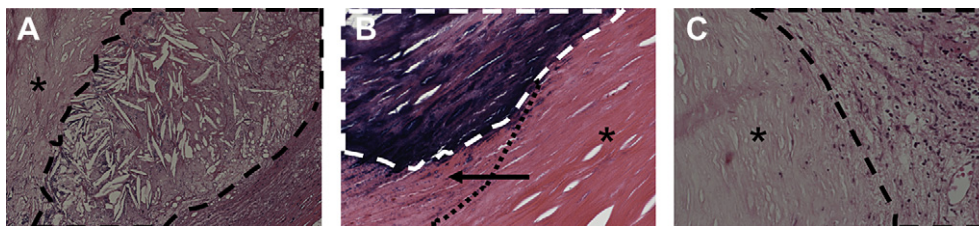


Figure 2. Morphological characterization of plaques was defined by four categories. *Lipid* – obvious confluent areas of lipid deposition whether intra- or extra-cellular. *Calcification* – obvious measurable areas of confluent calcification. *Fibroconnective tissue* – obvious confluent areas of fibroconnective tissue deposition with no confluent areas of calcification, lipid or lymphocytic infiltration. *Fibroconnective tissue with obvious lymphocytic/macrophage cellular infiltrate* – obvious confluent areas of fibrointimal change with obvious lymphocytic or lymphocytic and macrophage inflammation throughout the area. Outlines in dashed lines encircle confluent zones of lipid (A), calcification (B), lymphocytic and macrophage infiltration (C), and fibroconnective tissue present in A, B and C as highlighted by asterisks. These were measured by tracing around the perimeter of confluent abnormality and subsequent image analysis. In Fig. 1B, an example of spotty non-confluent calcification (black arrow) is also present. This spotty calcification would be designated within the “fibroconnective tissue” category and not included within areas designated as confluent calcification. Original magnification (A) $\times 200$, (B & C) $\times 400$.

tap water. The DAKO G2 Double staining kit, K5361 (Dako) was used for staining with anti-CD68 and the M2-macrophage markers^{6,18–20} dectin-1 (R&D Systems, Abingdon, UK), SOCS1 (Abcam, Cambridgeshire, UK) and CD163 (AbD Serotec, Kidlington, UK) and the M1-macrophage markers,^{4,6,18} inducible nitric oxide synthase, iNOS (BD Biosciences, Cambridge, UK), MHC class II (Dako) and SOCS3 (Abcam), according to the manufacturer’s instructions; positive staining was detected using diaminobenzidine/liquid Permanent Red (Dako). A negative control where the primary antibody was replaced by a non-specific IgG equivalent was included in all analyses.

Quantification of T-cell and macrophage counts

Intact CD68-positive cells with well-defined cytoplasm and nuclei were counted in the entire plaque to obtain the absolute counts. Counts were repeated until 3 consecutive values were obtained with a margin of error of ± 10 cells and an average taken as the absolute macrophage count. The area of the plaque was estimated in mm^2 by photographing the plaque under a dissecting microscope at $0.75\times$ and using “Volocity” image analysis software (PerkinElmer Life and Analytical Sciences, Inc). Absolute counts were then divided by the plaque area to obtain the intact macrophage counts per mm^2 . A similar technique was used to count the CD3-positive T-cells per area. Both the intra-observer and inter-observer variation were found to be less than 5% ($P < 0.002$). Double stained sections were analyzed using bright-field light and positivity for liquid permanent red confirmed using rhodamine fluorescent microscopy. Macrophage-rich clusters within the plaque were identified and on average greater than fifty CD68-positive cells were counted. The number of these cells which were double positive was counted and expressed as a percentage of total macrophages. Preliminary quantification by two independent observers confirmed that the proportions of M1/M2 macrophages in macrophage-rich areas were not significantly different from the proportions estimated from total macrophage counts.

Statistical analyses

Comparison of demographic data was assessed by the Fisher’s exact test or two sample Student’s *t*-test. The Mann Whitney *U* test was used to compare the CD68 counts/ mm^2 , CD3 counts/ mm^2 , and morphological differences between the carotid and the femoral plaques. A Kruskal Wallis test was used to compare the markers of M1- and M2-activated macrophages between the carotid and femoral plaques. All values are reported as medians and interquartile ranges. Correlations were performed by Spearman’s method.

Results

Population characteristics

The demographic characteristics and risk factors profile for the patients undergoing carotid surgery and femoral artery surgery are shown in Table 1. Comparison of the two groups revealed no statistical differences in terms of age, gender, diabetes, hypertension, coronary heart disease, cholesterol lowering or anti-platelet therapy. A significantly higher proportion of patients who had femoral disease admitted to being smokers and were on anti-hypertensive therapy. All but one of the patients was on statin therapy. The median duration of statin therapy prior to surgery for the patients with symptomatic carotid disease was 4 weeks (interquartile range (IQR) 3–20 weeks). Four of these patients had

Table 1

Baseline demographics of patients with symptomatic carotid and femoral artery disease. Values show number of patients and percentage (%) in each group.

	Carotid plaques (n = 32)	Femoral plaques (n = 25)	P
Patient details			
Median age (IQR) years	69(48–83)	71(56–78)	0.51
Number of males (%)	21(66%)	18(72%)	0.78
Co-morbidities (%)			
Diabetes	7(22%)	6(24%)	0.99
Hypertension ^a	30(94%)	21(84%)	0.39
Coronary heart disease (Angina, MI)	14(44%)	16(64%)	0.18
Smoking	16 (50%)	23(92%)	0.001
Medication			
Cholesterol lowering therapy	32(100%)	25(100%)	1.0
Anti-platelet therapy	32(100%)	25(100%)	1.0
Anti-hypertensive therapy	12(38%)	15(60%)	0.11
Symptoms (number of patients)			
Transient ischemic attack	16(50%)		
Crescendo TIA	3(9%)		
Cerebrovascular accident	9(28%)		
Amaurosis fugax	4(13%)		
Short distance intermittent claudication		9(36%)	
Rest pain		11(44%)	
Ulceration/gangrene		5(20%)	
Duplex detected ipsilateral internal carotid artery stenosis; median (IQR)	80%(70–99%)		
Ipsilateral ankle brachial pressure index median of highest value (IQR)		0.52(0–0.95)	

^a Hypertension is defined as a history of systolic pressure >140 mmHg or a diastolic pressure >90 mmHg upon at least three repeated measurements.

Table 2

Differences between carotid and femoral plaque morphometry. Confluent areas were determined by morphometry as described in *Materials and methods*. Values show median area or counts/mm² and interquartile range in brackets from sections -3, 0 and +2. * marks statistically significant differences in area composition between femoral and carotid plaques.

Confluent area (mm ²)	Carotid plaques	Femoral plaques	P value
Lipid	2.63 (0–15.29)	0.184 (0–0.92)	0.012*
Calcification	0.19 (0.01–13.62)	11.14 (0.75–27.40)	0.143
Fibroconnective tissue	12.60 (0.15–57.39)	69.67 (34.7–98.93)	0.002*
Fibroconnective tissue with cellular infiltrate	64.60 (11.5–87.37)	1.04 (0–39.32)	0.002*
Macrophage counts	39.76 (34.20–49.96)	13.20 (7.80–21.13)	0.001*

previously been on statin therapy (maximum duration 20 weeks) and were on statin therapy at the time of their recent cerebrovascular event. The median time between index symptoms and carotid surgery was 4 weeks (IQR 2–10 weeks). The majority of the patients with femoral artery disease had been on statin therapy long term, apart from two which were started 6 weeks prior to surgery. The median duration of statin therapy prior to surgery for the patients with femoral artery disease was 66 weeks (IQR 52–105 weeks). All patients with femoral artery disease had been on anti-platelet therapy long term with mean duration prior to surgery 23 months (IQR 2–94 months) while for those with carotid disease the mean duration before surgery was 2 months (IQR 1–53 months).

Femoral and carotid endarterectomy specimens display different histopathology and plaque morphometric characteristics

All carotid and femoral plaque specimens were classified as unstable according to the modified AHA classification.¹⁶ The majority of lesions were classed as grade IV lesions in the three anatomical sections studied from each case. Grade IV lesions were

defined as a plaque with a lipid or necrotic core surrounded by fibrous tissue with possible calcification. In four of the carotid plaques, and one of the femoral plaques, luminal thrombosis was present (grade VI).

Confluent zones of plaque were defined by morphometry as in Fig. 2. The percentage area occupied by confluent lipid and areas with marked lymphocytic and monocyte/macrophage infiltrates were significantly greater in carotid plaques as compared to that of femoral plaques (Table 2). In contrast, areas of fibroconnective tissue were significantly greater in the femoral plaques. The percentage area of confluent calcification was elevated but was not statistically greater in the femoral plaques. The percentage area of collagen was significantly greater in femoral plaques, (median (IQR) 48.2% (30.1–66.8) versus 32.6% (21.2–44.9); Mann–Whitney U test, P < 0.01).

Numbers of macrophages and T-cells per plaque area differ between femoral and carotid endarterectomy specimens

There was no significant difference between the total numbers of CD68-positive macrophages or CD3-positive T-cells in each of the three segmental sites assessed within the carotid or femoral plaques (Fig. 3A and B) reflecting plaque homogeneity. The CD68-positive intact macrophage counts were significantly higher in carotid as compared to femoral plaques, consistent with morphometric analysis, (median (IQR) 39.76 (34.2–49.96) versus 13.20 (7.8–21.13) counts per mm²; Kruskal Wallis test, P < 0.001), Fig. 3. Likewise, the CD3-positive T-cell counts per mm² were significantly higher in carotid plaques, (10.62 (7.89–13.67) versus 3.15 (2.0–5.7); Kruskal Wallis test, P < 0.001).

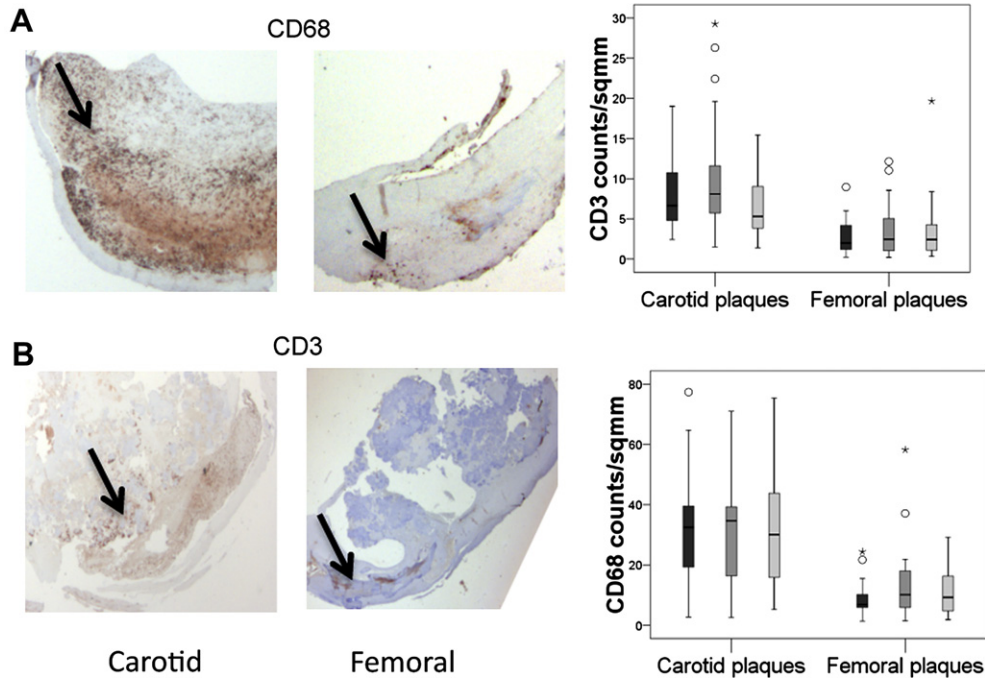


Figure 3. Carotid artery plaques show significantly higher numbers of total CD3-positive T cells (A) and total CD68-positive macrophage (B) per unit area than that of femoral artery plaques (denoted by arrows). Box plots show the median numbers of cells per area (mm²) of carotid (n = 32) and femoral (n = 26) plaques. The box represents the inter-quartile range (IQR) and the whiskers represent the range (95% confidence interval). The circles are outliers which are 1.5 IQR from the end of the box and the stars are outliers which are 3IQRs from the end of a box. The three bars within carotid or femoral plaque groups represent stained section 0 at the bifurcation, section -3 representing 2 sections distal to bifurcation and section +2 representing 1 section proximal to the bifurcation, as described in *Materials and methods*. Original magnification for Figures ×200.

The proportions of M1- and M2-macrophage subtypes differ between femoral and carotid endarterectomy specimens

M1-macrophages

The median percentage of iNOS-positive, CD68-positive M1-macrophages was significantly increased in the carotid compared to femoral plaques (median (IQR) 79% (72–83) versus 58% (51–62); Kruskal Wallis test; $P < 0.001$) as was the median percentages of MHC class II-positive, CD68-positive macrophages (74% (71–81) versus 40% (34–48); $P < 0.001$), Fig. 4. The percentages of SOCS3-positive, CD68-positive macrophages were also significantly increased in the carotid compared to the femoral plaques (76% (71–82) versus 44% (30–54); $P < 0.001$).

M2-macrophages

The median percentage of dectin-1-positive, CD68-positive M2-macrophages was significantly decreased in the carotid compared to femoral plaques, (median (IQR) 22% (15–33) versus 52% (46–57); $P < 0.001$) as were the percentages of CD163-positive, CD68-positive macrophages (26% (17–35) versus 60% (55–76); $P < 0.001$). Similarly, the percentages of SOCS1-positive, CD68-positive M2-macrophages were significantly decreased in the carotid compared to femoral plaques (16% (9–28) versus 42% (36–53); $P < 0.001$).

Correlation between immunohistochemistry and morphometry

The maximum number of CD68-positive macrophage counts correlated negatively with the median percentage area of fibroconnective tissue for the three sections examined (Spearman's correlation; $R_s = -0.282$; $P = 0.039$) and collagen ($R_s = -0.447$; $P = 0.001$) but positively with fibroconnective tissue with obvious lymphocytic cellular infiltrate ($R_s = 0.332$; $P = 0.014$). No significant correlation was observed between total macrophage counts and percentage area of lipid core or calcification.

The expression of M1-macrophage markers correlated with fibroconnective tissue with obvious cellular infiltrate, ($R_s = 0.379$; $P = 0.005$) and the correlation was greater than that of total macrophages, whereas M2-markers exhibited a negative correlation ($R_s = -0.292$; $P = 0.033$). In contrast to total CD68+ macrophages, the percentage of M1-markers correlated significantly with the proportion area of confluent lipid ($R_s = 0.353$; $P = 0.03$) and the percentage of M2-markers correlated significantly with areas of calcification ($R_s = 0.304$; $P = 0.031$). Within the carotid and femoral plaques there was no significant correlation between the total numbers of CD68-positive macrophages and duration of statin therapy (weeks). However, proportions of M1 macrophages showed a significant negative correlation with duration of statin therapy ($R_s = -0.506$, $P < 0.001$) while M2 macrophages showed a positive correlation ($R_s = 0.493$, $P < 0.001$). By contrast, there was no significant correlation between total numbers of macrophages or proportion of macrophage subsets and the duration of anti-platelet therapy.

Discussion

This study has demonstrated important associations of the percentage of M1- and M2-macrophages with compositional features of the plaque that, to date, have been lacking in human atherosclerosis studies.^{21,22} Carotid and femoral plaques exhibit substantially different cellular and morphological compositions as may be anticipated from their location and clinical presentations. By using a panel of markers and co-staining for CD68 positive cells we show a greater preponderance of M1-macrophages within carotid compared to femoral plaques and relate this to morphometrically measured plaque components.

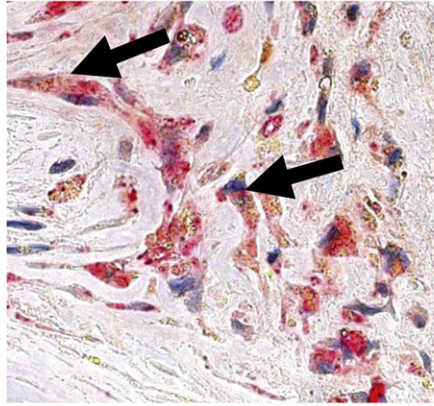
M1-macrophages are considered to have a potential role in atherogenesis and plaque instability due to their high expression of metalloproteinases, cytokines, bioactive lipids and tissue destructive nitrogen and oxygen radicals.^{4–6} Consistent with the greater proportion of M1-macrophages in carotid plaques, we show, using detailed morphometric analysis, that carotid plaques exhibit significantly greater percentage areas of confluent lipid and immune-cell infiltrate and show reduced cap thickness compared to femoral plaques. These features suggest that M1-macrophages are a further identifiable feature of the unstable plaque.

M2-macrophages have potential to promote tissue reparative processes and enhance plaque stability due to their well reported immunoregulatory, reparative phenotype and ability to promote collagen synthesis.^{4–6,23} In line with this we show a greater percentage area of collagen in M2-macrophage rich femoral plaques than in M2-macrophage poor carotid plaques. For femoral plaques, percentage areas of collagen and fibroconnective tissue were significantly increased and confluent calcification tended to be higher. Plaques from both the common and superficial arteries were included in the femoral group. Plaque composition between the two is reported to differ²⁴ yet when analyzed together in our study, the composition was still significantly different from that of symptomatic carotid plaques. Femoral plaques have previously been reported to exhibit significantly more calcification than carotid plaques¹² and increased levels of plaque calcification has been observed in asymptomatic as compared to symptomatic carotid plaques.²⁵ We would suggest that the presence of calcium within plaques relates to stable plaques rather than defining unstable plaques, as suggested previously.²⁶

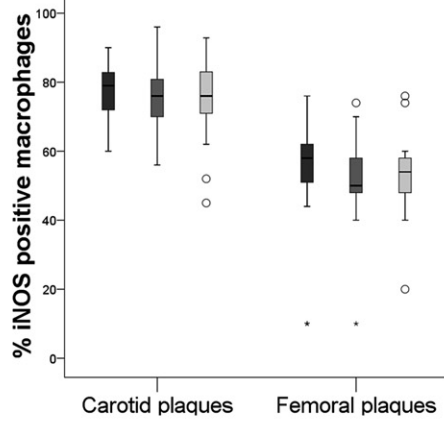
M1- and M2-macrophage markers in single stained sections has been previously demonstrated in distinct locations of lipid-rich human carotid atherosclerotic lesions.²¹ However, MCP-1, the only M1-marker used in this previous study, is not specific to macrophages and is largely secreted. Moreover, no correlations were performed on proportions of M1- or M2-macrophages and plaque phenotype, and lipid poor plaques were not evaluated. The current study is the first to definitively characterize macrophage phenotype by the co-localization of CD68-positive cells with a panel of M1- and M2-macrophage markers which is critical for a specific and comprehensive analysis of the proportion of macrophage types within the plaque. This is not possible by total mRNA or protein levels in plaque that would be strongly dependent on the number of macrophages present in tissue and expression levels by cells other than macrophages. Importantly, correlations between M1-macrophages and morphological parameters linked to plaque instability (lipid, lymphocytic cellular infiltrate) or M2 macrophages and features of stability (calcium, fibroconnective tissue)² was more highly significant than correlations with total macrophage counts across all plaques studied. This supports the view that the proportion of macrophage subsets within plaques is a better predictor of the compositional phenotype and related stability of plaque as compared to total macrophages.

The difference in duration of statin therapy between patients with carotid and femoral disease is a confounding variable and could account for some decrease in lesion inflammation in femoral plaques. Total macrophage counts within plaques did not correlate significantly with the duration of time the patient was on statin therapy, yet, we show for the first time, a significant negative correlation between duration of statin therapy and proportion on M1-macrophages. There is no evidence that statins can switch plaque macrophages from an M1 to an M2 phenotype but they could influence the initial polarization to M2 by dampening the inflammatory environment.^{27,28} Anti-platelet therapy could also exert beneficial effects on the inflammatory composition of human atherosclerotic carotid plaques but we did not find a correlation

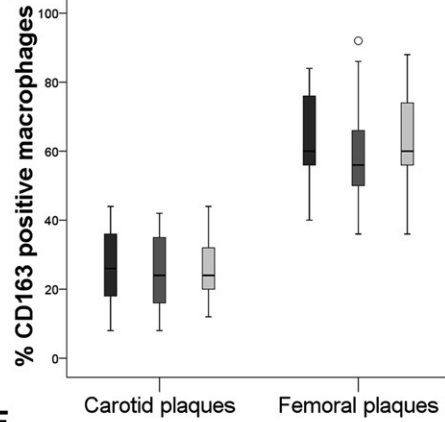
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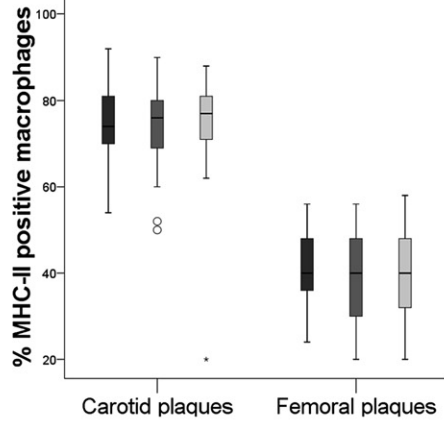
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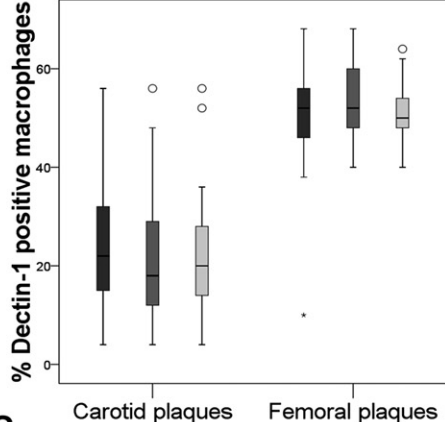
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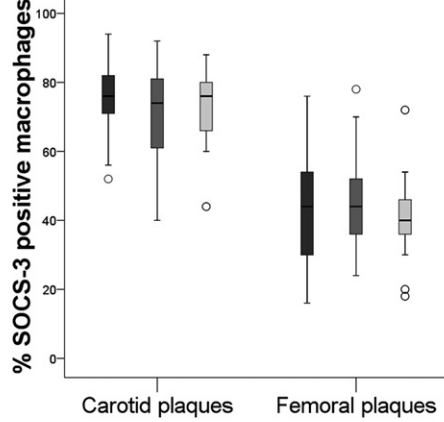
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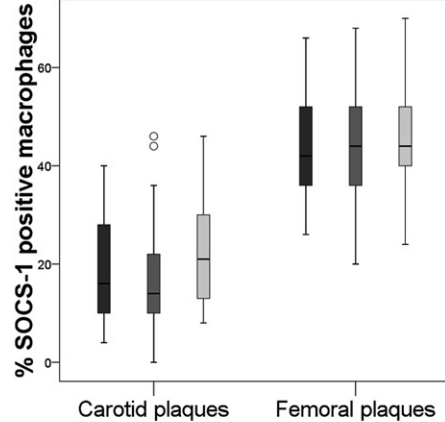
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between duration of anti-platelet therapy and total macrophages or their subsets in our study.

In summary this study has shown a relationship between the phenotype of macrophages subsets and the compositional markers of plaque stability in two distinct anatomical atherosclerotic arterial beds. In future studies it would be of value to compare macrophage subsets in symptomatic versus asymptomatic plaques. It may also be of value to develop ligands against M1-macrophages for positron emission tomography (PET) imaging with the aim of non-invasively identifying the unstable plaque.

Appendix A. Supplemental material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ejvs.2012.08.005>

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Conflict of Interest

None.

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Figure 4. Double immunostaining of endarterectomy sections showing a greater predominance of M1-activated macrophages in carotid plaques and of M2-activated macrophages in femoral plaques. Total macrophages were detected by anti-CD68 and liquid permanent red staining (red) while macrophage activation markers were identified by the relevant antibodies and DAB staining (brown). Macrophage-rich areas were analyzed as outlined in Methods. Examples of double stained macrophages are highlighted by arrows in (A). Box plots show median values for numbers of M1-macrophages iNOS (B), MHC class II (C), SOCS3 (D) or M2-macrophages, CD163 (E), Dectin1 (F), and SOCS1 (G) in femoral or carotid plaques. The box represents the inter-quartile range (IQR) and the whiskers represent the range (95% confidence interval). The circles are outliers which are 1.5 IQR from the end of the box and the stars are outliers which are 3IQRs from the end of a box. The three bars grouped within carotid or femoral plaques represent stained sections 0, section –3 and section +2 representing 1 section proximal to the bifurcation, as described in *Materials and methods*.