Multi-analyte profiling in human carotid atherosclerosis uncovers pro-inflammatory macrophage programming in plaques

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Summary

Molecular characterisation of vulnerable atherosclerosis is necessary for targeting functional imaging and plaque-stabilising therapeutics. Inflammation has been linked to atherogenesis and the development of high-risk plaques. We set to quantify cytokine, chemokine and matrix metalloproteinase (MMP) protein production in cells derived from carotid plaques to map the inflammatory milieu responsible for instability. Carotid endarterectomies from carefully characterised symptomatic (n=35) and asymptomatic (n=32) patients were enzymatically dissociated producing mixed cell type atheroma cell suspensions which were cultured for 24 hours. Supernatants were interrogated for 45 analytes using the Luminex 100 platform. Twenty-nine of the 45 analytes were reproducibly detectable in the majority of donors. The in vitro production of a specific network of mediators was found to be significantly higher in symptomatic than asymptomatic plaques, including: tumour necrosis factor α, interleukin (IL) 1β, IL-6, granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage colony-stimulating factor (M-CSF), CCL5, CCL20, CXCL9, matrix metalloproteinase (MMP)-3 and MMP-9. Ingenuity pathway analysis of differentially expressed analytes between symptomatic and asymptomatic patients identified a number of key biological pathways ($p<10^{-25}$). In conclusion, the carotid artery plaque culprit of ischaemic neurological symptoms is characterised by an inflammatory milieu favouring inflammatory cell recruitment and pro-inflammatory macrophage polarisation.

Keywords

Atherosclerosis, carotid stenosis, inflammation, cytokines

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Introduction

Atherosclerosis shares many biological features with other diseases caused by chronic inflammation (1). Genetic ablation or intervention studies in mouse models of atherosclerosis have mapped the cellular components of the plaques' inflammatory infiltrates, and the soluble mediators necessary for their recruitment and activation (2).

There is less knowledge on human atherosclerotic disease, and even less on the mechanisms that make the human plaque prone to thrombo-embolic complications. Anatomo-pathological evidence links inflammation with disease activity in humans. The number of monocyte-macrophages infiltrating the plaque (3) is related to plaque vulnerability, and the inflammatory infiltrate is abundant at sites of plaque erosion and rupture (4, 5). Moreover, lymphocyte numbers and their activation markers relate to plaque activity (5).

Macrophages are plastic and capable of modifying their behaviour upon micro-environmental cues. Classic (or M1) 'activation' in response to bacterial motifs such as lipopolysaccharide (LPS) and interferon (IFN)γ mirrors Th1 lymphocyte polarisation. M1 macrophages produce pro-inflammatory cytokines such as interleukin (IL)1, IL12 and tumour necrosis factor (TNF)α (6). Alternative (or M2) macrophages are heterogeneous and can be generated by different inflammatory and opsonic signals (7). Both IFNγ and Toll-like receptor 4 (TLR4, the LPS receptor) are implicated in M1 polarisation and their genetic deletion reduces atherosclerosis development (8, 9). M2 macrophages accumulate first in murine atherosclerosis, while lesion progression correlates with predominance of M1 over M2 macrophages (10). In murine plaque oxidised phospholipids induce heme oxygenase-1 (HO-1)-expressing "Mox" macrophages (4). Exposure of the vessel wall to low shear stress is associated with M1-type macrophage polarisation (11).

Several studies have assessed the expression in human atherosclerotic plaques of several intracellular and surface markers of M1 and M2 polarisation. The results show a mixture of M1 and M2 macrophages (12, 13) with a segregation of the two phenotypes within different part of the plaque, with M1 activation predominating in rupture-prone plaque areas (12). The M1/M2 macrophage ratio in human epicardial fat samples correlates with the severity of coronary artery disease (14). Intraplaque haemorrhage-derived heme induces Mhem macrophages expressing CD163 and HO-1. Carotid atherosclerosis compared to femoral plaques, displays a predominance of M1 macrophages (15). However, the macrophage polarisation linked to symptomatic carotid atherosclerosis versus asymptomatic is uncertain, limiting the identification of disease biomarkers.

Human diseased tissue holds the clues to human disease pathogenesis. We have previously established a methodology for the isolation of viable cells from human carotid atherosclerotic lesions, a model system that attempts to reproduce the complex cellular interactions *ex vivo*. As previously published, the mixed cell population contains macrophages, lymphocytes and smooth muscle cells, and displays cytokine, chemokine and matrix metalloproteinase (MMP) production in absence of extrinsic stimulation (16, 17). Inflammatory molecule production has been shown to be dependent upon TLR2 signalling (17) and the nuclear factor-κB (NFκB) pathway via myeloid primary differentiation response gene 88 (MyD88) (16). Whether this *in vitro* cytokine production is affected by the symptomatic status of the patients is uncertain. We identify here a specific network of secreted mediators associated with the high-risk carotid plaque and their clustering. Our findings suggest that live atheroma cells isolated from carotid plaques that gave rise to neurological symptoms maintain *in vitro* a pro-inflammatory programming that on the whole supports M1-type macrophage polarisation.

Table 1: Demographic and clinical information relating to the study population. Data is presented as median (inter-quartile range), or number (%).

	Asymptomatic (n=32)	Symptomatic (n=35)
Age (years)	70 (60–78)	70 (67–74)
Male gender	26 (81 %)	23 (72 %)
Family history of arterial disease	9 (28 %)	6 (19 %)
Smoking	18 (56 %)	17 (53 %)
Hypertension	27 (84 %)*	21 (66 %)
Diabetes mellitus	4 (13 %)	11 (34 %)
Dyslipidaemia	20 (63 %)	24 (75 %)
Serum cholesterol (mmol/l)	3.6 (3.4–4.5)	3.7 (3.3–4.4)
Aspirin	24 (75 %)	27 (77 %)
Statin	28 (88 %)	33 (94 %)
ACEi or A2RA	20 (63 %)	20 (57 %)
Plaque carotid luminal stenosis	81 (75–90)	88 (80–93)
Time from symptoms to carotid endarterectomy (days)	N. A.	20 (12–41)

* There were statistically significantly more patients with hypertension in the asymptomatic group compared to the symptomatic group (p=0.0329; Fisher's exact test). ACEi, angiotensin converting enzyme inhibitor; A2RA, angiotensin 2 receptor antagonist; N. A., not applicable.

Methods

Patient population, sample and data collection

Atherosclerotic plaques were collected from consenting patients undergoing carotid endarterectomy. Patients were considered symptomatic if they experienced, within the preceding six months, ipsilateral carotid territory focal neurological symptoms (stroke, transient ischaemic attack or amaurosis fugax). Symptomatic status was confirmed by a neuro-vascular multi-disciplinary team. Research ethics committee approval was obtained (RREC2989, 08/H0706/129).

Ex vivo culture of cells isolated from human atherosclerotic plaques

Fresh diseased intimal arterial segments were dissected from carotid endarterectomy specimens under a dissecting microscope. Single cell suspensions were obtained by enzymatic digestion, as published (16, 17). Freshly isolated atheroma cells were cultured at 1×10^6 cells/ml in RPMI containing 10 % fetal bovine serum in at least three replicate wells (Biosera, Ringmer, UK). Viability was determined via Trypan Blue exclusion and propidium iodide (PI) after cell isolation and found > 95 % in all preparations. Viability in culture was monitored with 3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium (Sigma, Dorset, UK) (16, 18). LPS content in all media, reagents, and supernatants from experiments was assessed via the Limulus Amebocyte assay (Sigma) and only samples below detection limits were retained for analysis. Supernatants were removed after 24 hours and stored at –80 °C for single-batch analysis.

Multi-analyte profiling using the Luminex 100 platform quantified supernatant protein levels of cytokine and chemokine (Milliplex Human Cytokine / Chemokine Kit, Millipore Corporation, Billerica, MA, USA), MMP and tissue inhibitor of metalloproteinase (TIMP) (Fluorokine MAP Human Multiplex Kits, R&D Systems, Abingdon, UK). Each replicate culture was analysed in duplicate. Normalisation to global sample median was performed when comparing values across plates. Where an analyte level was below detection limits, it was ascribed the lowest standard value for statistical analysis.

Statistical analysis

Data was analysed with Prism (v6.0c, GraphPad, San Diego, CA, USA). Data was not normally distributed according to the D'Agostino and Pearson omnibus normality test. Data were log-transformed and the statistical analysis was performed using unpaired

Table 2: Cytokine and chemokine levels differ in mixed cell culture from symptomatic compared with asymptomatic atherosclerotic plaques. Data are presented as median and interquartile range. Statistical analysis was performed using unpaired Student t-test after a logarithmic transformation. P-values were corrected using the Benjamini-Hochberg False Discovery Rate (FDR) method. Benjamini and Hochberg corrected significance level (q*) was 0.0172414. True discovery (p<q*) is indicated with a star.

Analyte	Detected (N= 67)	Asymptomatic (n=32)	Symptomatic (n=35)	P-value	q*	Discovery
		Median (Inter-Quartile Range) (pg/ml)				p<q*
CCL5	65	43.8 (26.9–83.1)	120.5 (56.4–203.4)	0.0011	**0.0017**	*
CCL20	45	9.8 (9.8–25.3)	53.1 (14.6–100.7)	0.0018	**0.0034**	*
CXCL9	51	80.6 (48.8–224.9)	252.7 (112.2–400)	0.0021	**0.0052**	*
MMP-9	66	3456 (1309–8011)	8391 (3799–22050)	0.0037	**0.0069**	*
GM-CSF	64	69.3 (21.9–241.4)	246.7 (95.2–433.1)	0.0069	**0.0086**	*
IL1	58	16.6 (4–67.6)	72.9 (20.4–263)	0.0079	**0.0103**	*
IL6	67	1790 (579.2–4180)	8398 (1463–9892)	0.0087	**0.0121**	*
TNF	65	148.1 (61.7–319.5)	436.3 (147.7–1032)	0.0105	**0.0138**	*
MMP-3	36	16.1 (16.1–131.2)	118.2 (16.1–341.9)	0.0111	**0.0155**	*
M-CSF	63	330.9 (180.1–586.7)	552.1 (329.4–846.4)	0.0112	**0.0172**	*
sCD40L	26	13.9 (4.8–25)	31.6 (14.5–71.3)	0.0286	0.0190	-
CXCL10	60	13.9 (4.8–25)	31.6 (14.5–71.3)	0.0286	0.0207	-
MMP-1	64	625 (182.3–2007)	1753 (433.4–8569)	0.0318	0.0224	-
IL10	63	62.2 (13.7–274)	225.9 (48–572.3)	0.0326	0.0241	-
CCL2	67	318.3 (121.3–1050)	1180 (369.4–3757)	0.0417	0.0259	-
IL1	45	3.4 (3.2–36.5)	16.6 (6.1–45)	0.0431	0.0276	-
MMP-8	47	404 (91.9–1067)	616.6 (378–1903)	0.0490	0.0293	-
CCL14	67	34.5 (25.4–66.1)	62.4 (29.6–86.3)	0.1057	0.0310	-
CXCL7	67	118.9 (64.2–271.9)	178.7 (97.3–386.5)	0.1075	0.0328	-
MMP-7	37	255.5 (102.2–1016)	686.8 (102.2–2129)	0.1497	0.0345	-
TIMP-2	67	5829 (3829–131400)	42560 (3870–119500)	0.3321	0.0362	-
MMP-12	67	480.3 (257.4–1322)	666.7 (272.3–1278)	0.3468	0.0379	-
IL4	38	3.4 (3.2–10.2)	7.3 (3.2–9)	0.3808	0.0397	-
IFN 2	26	3.2 (3.2–7.7)	3.2 (3.2–9.5)	0.4621	0.0414	-
TIMP-1	67	4451 (2936–9697)	4014 (2201–9970)	0.4676	0.0431	-
VEGF	51	35.7 (3.2–62.7)	38.3 (16.67.2)	0.6236	0.0448	-
IL12 (p40)	38	6.9 (3.2–29.8)	9.6 (3.2–18.7)	0.6314	0.0466	-
MMP-2	58	679.3 (511.2–1284)	892.7 (496.8–1291)	0.8844	0.0483	-
CX3CL1	59	16 (9.6–20.7)	16 (9.6–18.4)	0.9550	0.0500	-
CCL19	0	-	-	-	-	
CXCL6	9	9.8 (9.8–9.8)	9.8 (9.8–9.8)	-	-	
CXCL11	6	2 (2–2)	2 (2–2)	-	-	
IFN	8	3.2 (3.2–3.2)	3.2 (3.2–3.2)	-	-	
IL2	2	3.2 (3.2–3.2)	3.2 (3.2–3.2)	-	-	
IL5	0	-	-	-	-	
IL11	0	-	-	-	-	
IL12 (p70)	3	3.2 (3.2–3.2)	3.2 (3.2–3.2)	-	-	
IL15	2	3.2 (3.2–3.2)	3.2 (3.2–3.2)	-	-	
IL17	1	-	3.2 (3.2–3.2)	-	-	
IL29	0	-	-	-	-	
TNF	0	-	-	-	-	
XCL1	3	19.5 (19.5–19.5)	19.5 (19.5–19.5)	-	-	
MMP-13	3	86.4 (86.4–86.4)	86.4 (86.4–86.4)			
TIMP-3	9	155 (155–155)	155 (155–155)			
TIMP-4	3	-	6.2 (6.2–6.2)	-	-	

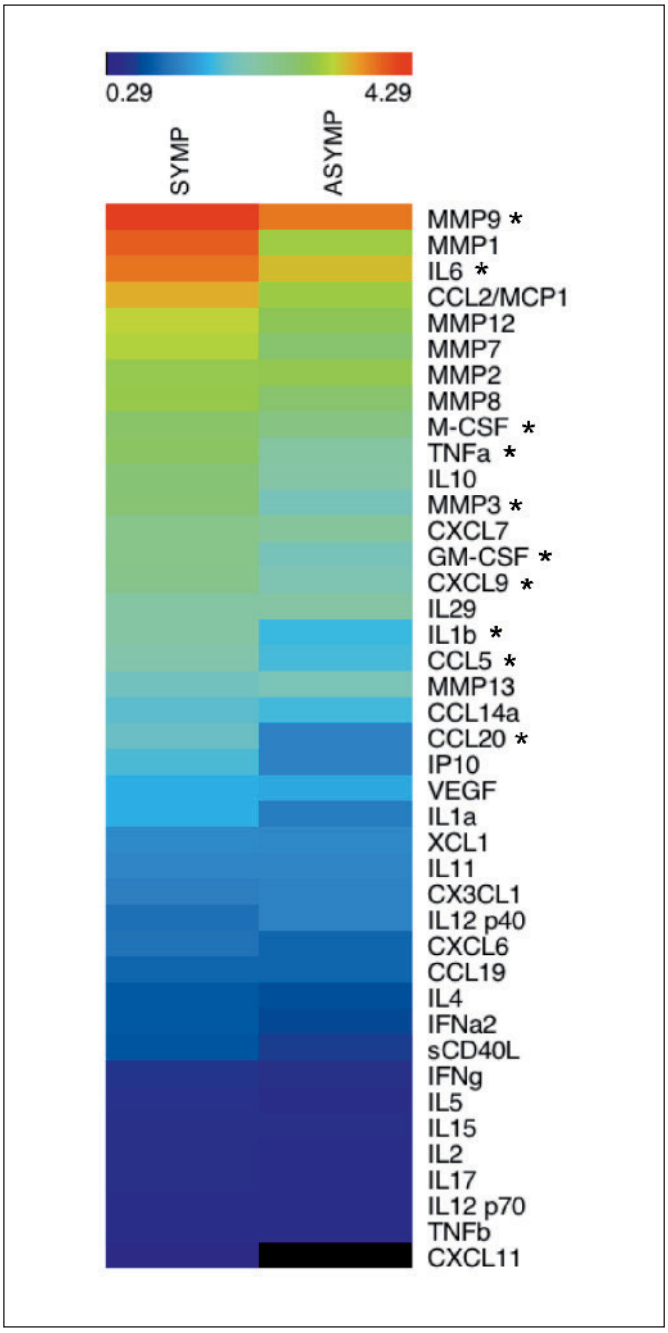

Figure 1: The ex vivo production of pro-inflammatory mediators differs between cells isolated from the carotids of symptomatic and asymptomatic subjects. Cells were isolated from carotid plaques as a mixed suspension, cultured at 10^6 cells/ml and supernatant collected at 24 hours, then aliquoted and frozen at –80 °C for single batch analysis. In the absence of exogenous stimulation, cells displayed spontaneous production of pro-inflammatory mediators. Multi-analyte profiling was accomplished on a Luminex 100 platform. Samples were analysed in duplicate. Heat map representation of analyte levels. Comparing symptomatic with asymptomatic plaques, analyte levels that were statistically significantly different following correction for multiple comparisons are highlighted with an asterisk.

Student's t-test. P-values were corrected using the Benjamini-Hochberg False Discovery Rate (FDR) estimation procedure (q*) (19).

Pathway analysis

Differences in analyte concentrations between symptomatic and asymptomatic groups were converted to ratios. Resulting data sets were analysed using Ingenuity Pathway Analysis (Ingenuity Systems, version 7.6). A 1.5-fold cut-off value was set to identify proteins whose expression was significantly increased or decreased, creating a highly interconnected protein network. Biological functions and processes were attributed to predefined canonical pathways by mapping the network's proteins to functions in the Ingenuity ontology. A right-tailed Fisher's exact test was performed to determine the significance (p-value) of any over-representation of proteins to a function compared to the result expected by a random set of proteins. The protein interaction network was created using the String database (StringDB v10.0) of known and predicted protein-protein interactions and inferring protein associations from co-expression data, according to the standard network instructions provided with a medium stringency threshold of association (0.4) (20).

Results

Sixty-seven patients (32 asymptomatic, 35 symptomatic) underwent carotid endarterectomy. The asymptomatic group contained more individuals with a formal diagnosis of hypertension; the groups were otherwise well matched (►Table 1). There were no statistically significant relationships between these parameters and analyte levels, including no significant correlation between analyte levels and time from symptoms in the symptomatic group (data not shown).

We were able to reliably detect 29 of the 45 analytes (►Table 2) in the majority of donors. Analytes that were detectable in less than 10 patients were excluded from further analysis. There was a predominance of myeloid-derived over lymphoid-derived cytokines in keeping with the predominance of macrophages in the culture system, as previously published (16, 17). For instance, IFNγ, IL-2 and IL-17 were not detectable in the majority of donors, while traditional macrophage derived cytokines such as TNFα, IL1β and IL-6 were abundant in the majority of donors (►Figure 1).

Cytokines

There was statistically significantly higher production of classical pro-inflammatory cytokines from symptomatic compared with asymptomatic atherosclerosis, including TNF-α, IL1β, and IL6 (►Figure 1, ►Figure 2, ►Table 2).

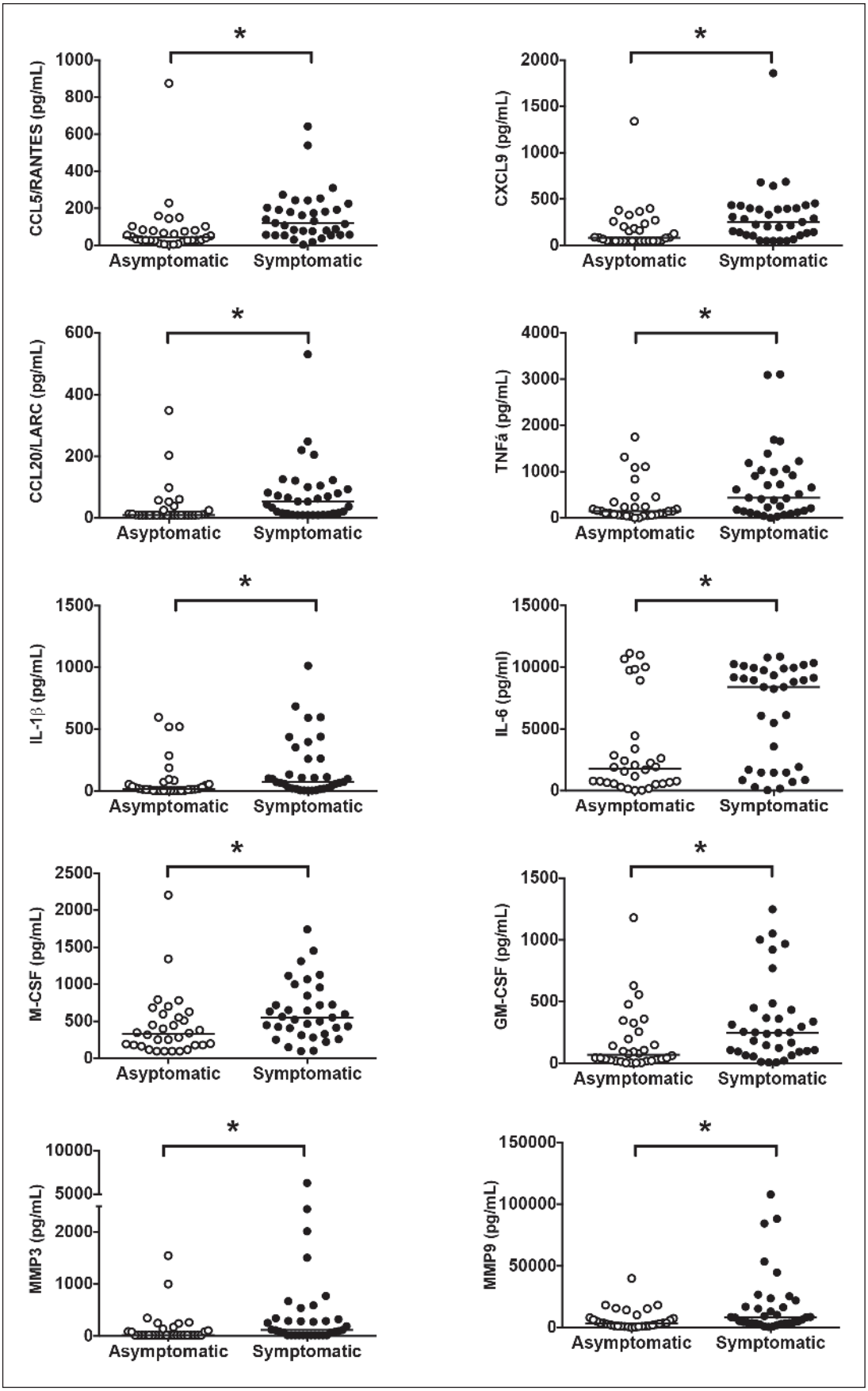

Figure 2: The production of 10 cytokine, chemokine and MMPs is higher in symptomatic vs asymptomatic carotid atherosclerosis. Cells were isolated from carotid atherosclerotic plaques as a mixed cell suspension and cultured at 1×10^6 cells/ml for 24 hours in the absence of any stimulus. Supernatants were collected, aliquoted and frozen at –80 °C for single batch analysis. Multi-analyte profiling was accomplished on a Luminex 100 platform. Following correction for multiple comparisons, levels of cytokine, chemokine, colony stimulating factor and matrix metalloproteinase production *in vitro* were significantly higher in atheroma cells from symptomatic (n=35) vs asymptomatic (n=32) plaques for the mediators shown. Each dot represents a single donor; medians are shown as dark horizontal lines; * $p < 0.05$.

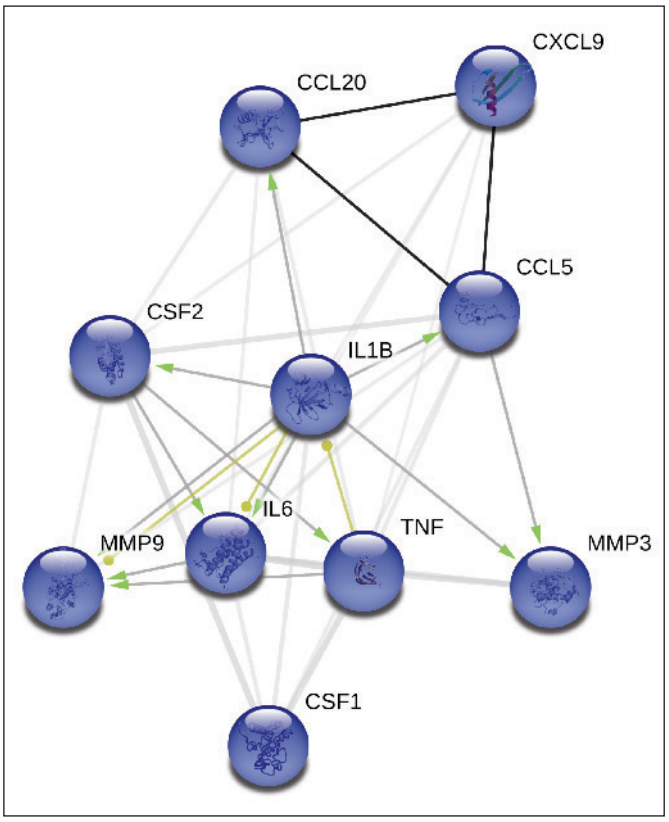

Figure 3. The interactions between the 10 analytes whose secretion was statistically significantly different between symptomatic and asymptomatic plaques, following correction for multiple comparisons.

Chemokines

CCL5, CCL20 and CXCL9 production was statistically significantly higher in symptomatic compared with asymptomatic atheroma culture. The latter two chemokines are known to be induced by IFNγ. Of note, IFNγ was detected in eight of the 67 cultures (►Figure 1, ►Figure 2, ►Table 2) but no difference was demonstrated on the basis of symptomatic status.

Colony-stimulating factors

Levels of production of both granulocyte-macrophage colony-stimulating factor (GM-CSF) and macrophage colony-stimulating factor (M-CSF) were statistically significantly higher in cultures obtained from symptomatic patients (►Figure 1, ►Figure 2, ►Table 2).

Matrix metalloproteinases (MMPs)

MMP-3 and MMP-9 levels were statistically significantly higher in symptomatic plaques compared with the asymptomatic plaques (►Figure 1, ►Figure 2, ►Table 2). MMP-13 production was undetectable in the majority of donors.

Tissue inhibitors of metalloproteinases (TIMPs)

There was no significant difference in the production of TIMPs between symptomatic and asymptomatic atherosclerosis (►Figure 1, ►Table 2). TIMP3 and TIMP4 was undetectable in the majority of donors.

Analyte inter-relationships and pathway analysis

The inter-relationship between analytes was computed statistically and via the use of Ingenuity pathways. Statistical analysis revealed significant relationship between analytes (Suppl. Figure 1, available online at www.thrombosis-online.com), for example the concentrations of classical pro-inflammatory cytokines TNFα, IL1α and IL1β were positively correlated with the IFNγ-dependent chemokine CXCL10 ($p<0.001$), colony stimulating factors GM-CSF ($p>0.001$) and M-CSF ($p<0.001$) and also with IL-10 production ($p>0.001$). Pro-inflammatory cytokine levels correlated positively with those of catabolic enzymes MMP-1 ($p<0.001$), MMP-3 ($p<0.001$) and MMP-9 ($p\leq0.005$).

The detectable analytes were identified within seven networks using Ingenuity Pathway Analysis (►Figure 4; Suppl. Table 1, available online at www.thrombosis-online.com). Canonical pathways associated with differences between symptomatic and asymptomatic datasets are shown in Suppl. Figure 2, available online at www.thrombosis-online.com, the most statistically significant pathways being: role of cytokines in mediating communication between immune cells ($p=9.64\times10–26$); altered T and B cell signalling in rheumatoid arthritis ($p=5.46\times10–23$); communication between innate and adaptive immune cells ($p=1.27\times10–22$); role of hypercytokinaemia / hyperchemokinaemia in the pathogenesis of influenza ($p=1.88\times10–22$); role of macrophages, fibroblasts and endothelial cells in rheumatoid arthritis ($p=2.37\times10–17$); atherosclerosis signalling ($p=4.58\times10–16$); and T helper cell differentiation ($p=5.37\times10–14$).

Discussion

Investigation of the molecular imprint of high-risk human atherosclerosis is needed for risk stratification (including biomarkers) and cardiovascular therapy, where soluble mediators are often ideal candidates. Hence, we set to characterise the inflammatory microenvironment of the high-risk human carotid plaque by using multianalyte profiling of soluble mediators. We found that human carotid plaques culprit of ischaemic neurological symptoms harbour a pro-inflammatory microenvironment that may favour the recruitment of Th1 lymphocytes and monocytes, and the generation of pro-inflammatory macrophages.

Our data clearly shows that atheroma cells derived from culprit carotid plaques exhibit a statistically significantly higher production of a highly interconnected network of cytokines and chemokines including TNFα, IL1β, IL-6, GM-CSF, M-CSF, CCL5, CXCL9 and CCL20. The classical triad of pro-inflammatory cytokines TNFα, IL1β and IL-6 is differentially released between cell

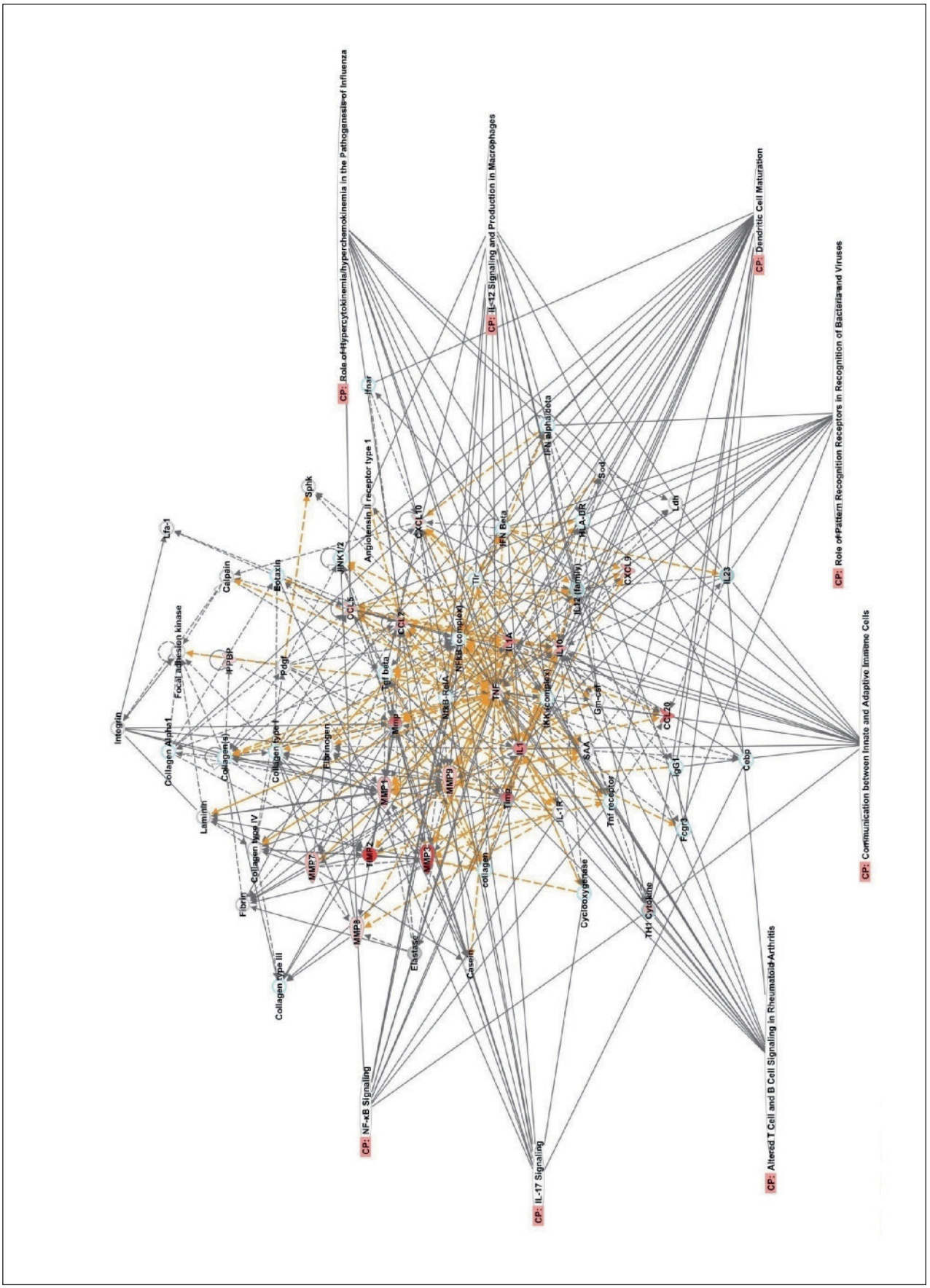

Figure 4: The inter-relationship between cytokines, chemokines and matrix metalloproteinases as constructed through application of a bioinformatic approach with Ingenuity Pathway Analysis. Key canonical pathways (CP) are highlighted at the periphery of the network.

What is known about this topic?

- Atherosclerosis is an inflammatory condition and atherosclerotic plaques become unstable in the context of pro-inflammatory cytokines and chemokines.
- Inflammatory molecule production has been shown to be dependent upon toll-like receptor 2 signalling, and the nuclear factor- B pathway via myeloid primary differentiation response gene 88.

What does this paper add?

- Symptomatic carotid atherosclerosis is characterised by an inflammatory milieu consistent with the predominance of pro-inflammatory M1-type macrophage polarisation.
- IFN signatures are observed, including significant elevation of CCL20, in symptomatic carotid atherosclerosis.
- Anti-inflammatory and anti-atherogenic cytokines, such as IL-10, attempt to offer regulation within the unstable plaque microenvironment.

preparations derived from patient with or without neurological symptoms, indicating that these classic macrophage-derived mediators have a prominent place in the pro-inflammatory milieu of the high-risk atherosclerotic plaque.

Many of the differentially secreted analytes are chemokines: CCL5, CXCL9, and CCL20, indicating that the cells from symptomatic carotid plaque support the recruitment of monocytes and Th1 cells (21). The three IFNγ-inducible CXC chemokines – CXCL9, CXCL10, CXCL11 – have been detected by atheroma-associated cells, as well as the expression of their receptor, CXCR3, by all T lymphocytes within human atherosclerotic lesions. CCL20 is associated with lymphoid neogenesis in human aortic aneurysms (22). The deletion of its receptor CCR6 in atheroprone mice reduces monocyte-mediated inflammation during atherogenesis (23). Importantly, CCL20 is also up-regulated by IFNγ-mediated human macrophage polarisation (24) and murine M1 polarisation (25). In support of this, we confirmed the production of CXCL10 (also IFNγ-dependent) and CCL20 to be positively correlated. Overall, the expression of IFNγ-inducible chemokines in our study could represent an IFNγ 'signature' that marks the high-risk carotid plaque. However, we were unable to consistently detect IFNγ production by plaque cells. It is likely that IFNγ production is confined to the early instability phase and only its late effectors are detectable at the time of endarterectomy. Supporting our observations, circulating monocytes isolated from patients with unstable angina, compared to chronic stable angina, exhibit signatures of IFNγ activation in the absence of elevated systemic levels of the cytokine (26).

Importantly, this cytokine and chemokine signature of symptomatic compared to asymptomatic carotid atherosclerosis complies largely with the cytokine and chemokine signature for M1 polarisation described by Martinez et al. in the transcriptional profiling of human monocyte-to-macrophage polarisation (24). LPS- and IFNγ-differentiated M1 macrophages had increased gene expression of TNF (21 fold), IL6 (7 fold), CXCL10 (59 fold), CXCL9 (58 fold), CCL5 (19 fold) and CCL20 (7 fold), than IL4-differentiated M2 macrophages (24). A similar pattern has been observed when comparing murine macrophages (27). Our findings suggest that even after isolation, atheroma cells maintain *in vitro* a pro-inflammatory programming that resembles M1-type macrophage polarisation. In our study, both M-CSF and GM-CSF are increased in unstable plaque pointing to an important role for macrophage differentiation and activation in plaque instability. In particular GM-CSF is known to induce pro-inflammatory features in macrophages, including the production of inflammatory cytokines such as TNFα and IL6, and their involvement in disease (28). Indeed, GM-CSF levels in our study significantly correlate with IL1α, IL1β and TNFα, supporting similar mechanisms in atherosclerosis.

There is an inextricable link between inflammation and matrix degradation in atherosclerosis. We found MMP-3 and -9 secretion to be significantly higher within symptomatic plaques, as has previously been described (29). Moreover, the top network identified by pathway analysis is pertinent to matrix degradation. We have found MMP-3 and -9 concentrations to be positively correlated with GM-CSF, TNFα, IL1α, IL1β, IL6, IL10, CCL2, CCL5, CCL20 and CXCL10 protein production. In keeping with our findings, MMP-3 expression was higher in human monocytes polarised via IFNγ and LPS exposure (30). However, in this study, MMP-9 was not affected by polarisation (30), and different results have been obtained with murine macrophages (30).

A limitation of our study is the lack of matching immunohistochemistry and cell phenotyping data. When isolating live cells from carotid endarterectomies, cell numbers are the limiting factor for downstream analysis and the cellular composition could not be examined for each of the carotid plaques included in this study. We chose to focus on the multiparametric interrogation of the secretion of the soluble mediators via Luminex platforms to maximize the numbers of donors included in the study. However, in a previous study (31) we have shown that the *ex vivo* production of IL6, MMP-1 and -3 from atheroma cells was higher in patients whose carotid plaques displayed higher microbubble contrast retention during late-phase contrast-enhanced ultrasound and had greater CD68 and CD31 percentage area immunopositivity by quantitative immunohistochemistry. These data together with the current data support our conclusion that *ex vivo* cytokine production reflects to some extent the biology of the atherosclerotic plaque *in vivo*.

A second limitation of our study derives from the fact that the enzymatic dissociation of tissues to achieve a single cell suspension results in the loss of tissue specific factors, such as local hypoxia, extracellular matrix, as well as the position of cells within the lesions (e.g. core vs shoulder of the plaque). On the other hand, our approach has advantages compared to analyses on whole tissue lysates, including the assessment of cytokine release based on a standardised cell density (10^6 per ml) rather than per unit mass of tissue with an unknown cellular representation, and the quantification of cytokines actually released (with the potential for biological activity) rather than stored intracellularly or within the extra-

cellular matrix. Within its limitation, our model remains a useful tool to inform on the biology of human atherosclerosis.

Our data demonstrate that cells isolated from human atherosclerotic plaques retain *in vitro* the ability to spontaneously produce pro-inflammatory mediators and that a selected network of cytokine, chemokine and MMP production is released in greater amounts by cells derived from patients with previous neurological symptoms of ipsilateral carotid origin as compared to cells isolated from asymptomatic subjects. This cytokine, chemokine and MMP pattern is broadly consistent with an intraplaque milieu that favours pro-inflammatory macrophage polarisation and an IFNγ signature. Of note, differences in cytokine, chemokine and MMP expression were evident despite 61 of the 67 patients in this study being subject to long-term statin therapy. This suggests that tailored therapies targeting modulation of inflammation may deliver an advantage as compared to standard treatment. Our study also has implications for future identification of soluble biomarkers for diagnostic applications for high-risk atherosclerosis.

Conflicts of interest
None declared.

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