Gene and Protein Expression of Chemokine (C-C-Motif) Ligand 19 is Upregulated in Unstable Carotid Atherosclerotic Plaques

M.K. Salem a,*, H.Z. Butt b, E. Choke a, D. Moore b, K. West b, T.G. Robinson c, R.D. Sayers d, A.R. Naylor a, M.J. Bown a

a Vascular Surgery Group, Department of Cardiovascular Sciences, University of Leicester, Leicester LE2 7LX, UK
b Department of Histopathology, University Hospitals Leicester NHS Trust, Leicester LE1 5WW, UK
c Ageing and Stroke Medicine Group, Department of Cardiovascular Sciences, University of Leicester, LE2 7LX, UK

WHAT THIS PAPER ADDS
This paper is the first study use of a whole-genome microarray platform to assess markers of plaque instability based upon multiple criteria. CCL19 gene upregulation in unstable plaques highlights a future target for research, and its protein circulating biomarker could be used in clinical practice to identify patients at high risk of stroke.

Objective/Background: The aim was to investigate the expression of genes associated with carotid plaque instability and their protein products at a local and systemic level.

Methods: Carotid plaques from 24 patients undergoing carotid endarterectomy (CEA) were classified as stable or unstable using clinical, histological, ultrasound, and transcranial Doppler criteria, and compared using whole genome microarray chips. Initial results of differentially expressed genes were validated by quantitative reverse transcriptase polymerase chain reaction in an independent group of 96 patients undergoing CEA. The protein product of genes significantly differentially expressed between patients with stable and unstable plaques were analysed by plaque immunohistochemistry and serum protein quantification by enzyme-linked immunosorbent assay on a further independent cohort.

Results: Expression of chemokine (c-c-motif) ligand 19 (CCL19) was significantly upregulated in plaques from patients with clinically unstable disease (p < .001). Cathepsin G expression was upregulated in histologically unstable plaques (p = .04). Serum concentration of CCL19 was significantly higher in patients with clinically unstable plaques (p = .02). Immunohistochemical staining for CCL19 demonstrated positive staining in histologically and clinically unstable plaques (p = .03). CCL19 also co-localised with CD3+ T-cell lymphocytes in the core region, around where CCL19 was expressed.

Conclusions: CCL19 is significantly overexpressed in patients with unstable carotid atherosclerotic plaques and may be a possible novel biomarker for identifying high-risk patients in whom more urgent intervention may be indicated.

© 2016 European Society for Vascular Surgery. Published by Elsevier Ltd. All rights reserved.
Article history: Received 28 January 2016, Accepted 19 May 2016, Available online 3 August 2016
Keywords: Atherosclerosis, Carotid, CCL19, Genes, Stenosis, Stroke

INTRODUCTION
Stroke is the second leading cause of death in the Western world and the leading cause of permanent neurological disability. More than one-third of all strokes are caused by thromboembolism from unstable carotid atherosclerotic plaques. Instability is characterised by a large lipid core and plaque inflammation, leading to cap rupture, surface ulceration, and thrombus formation. The pathogenesis of plaque instability is poorly understood, but is thought to result from a combination of altered gene expression and local and systemic inflammation.2

Although evidence-based national and international guidelines exist for the treatment of carotid artery stenosis,3–8 these only assess grade/severity of stenosis and timing from the most recent clinical event and do not include measures of biological instability. The identification of these patients with unstable carotid plaques remains important because they may require more urgent carotid endarterectomy (CEA) to prevent imminent stroke.

Plaque instability is usually determined by a combination of clinical, physiological, and histological parameters with the highest risk of ischaemic stroke occurring in the first 14 days after an index event such as transient ischaemic attack.
(TIA).\textsuperscript{9,10} Spontaneous embolisation detected by transcranial Doppler (TCD) monitoring of the middle cerebral artery is associated with a significantly increased risk of early stroke.\textsuperscript{11} In addition, histological characterisation of the carotid plaque can also identify the high-risk subgroup, but there is poor correlation between preoperative imaging and postoperative plaque analysis.\textsuperscript{12,13}

Despite progress in the clinical management of patients with symptomatic carotid artery disease, the molecular mechanisms which lead to plaque instability remain poorly understood. Elucidation of the pathways leading to plaque instability and identification of markers of instability may permit the stratification and prioritisation of patients for surgical intervention. In addition, this may also identify targets for treatment to avoid surgery.

In this study, the aim was to determine gene expression profiles associated with plaque instability using whole-genome microarray analysis of plaques harvested during CEA and determine if the protein product of any differentially expressed genes were present in the plaque and/or circulation.

**METHODS**

**Study design**

To search for pathways associated with carotid plaque instability, a two-stage study of gene expression was conducted in carotid atherosclerotic plaques, followed with serum protein quantification and plaque immunohistochemical analysis of positive findings from the gene expression studies. The gene expression studies consisted of an initial discovery study using whole-genome expression arrays and a validation study in an independent cohort.

**Ethics**

The study was authorised by the local ethics committee. The National Research Ethics Service Research Ethics Committee reference is 07/H0406/151.

<table>
<thead>
<tr>
<th></th>
<th>Grade 0</th>
<th>Grade 1</th>
<th>Grade 2</th>
<th>Grade 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haemorrhage</td>
<td>No haemorrhage</td>
<td>Small haemorrhage</td>
<td>Large haemorrhage</td>
<td>–</td>
</tr>
<tr>
<td>Thrombus</td>
<td>No thrombus</td>
<td>Small thrombus</td>
<td>Large thrombus</td>
<td>–</td>
</tr>
<tr>
<td>Lipid core</td>
<td>No lipid core</td>
<td>Small lipid core</td>
<td>Large lipid core</td>
<td>–</td>
</tr>
<tr>
<td>Fibrous tissue</td>
<td>Very little fibrous tissue</td>
<td>Approx. 50% fibrous tissue</td>
<td>Predominantly fibrous tissue</td>
<td>–</td>
</tr>
<tr>
<td>Chronic plaque inflammation</td>
<td>None</td>
<td>Occasional cells or one group &gt; 50</td>
<td>2–5 groups &gt; 50</td>
<td>&gt; 5 groups &gt; 50 or 1 group &gt; 500</td>
</tr>
<tr>
<td>Chronic cap inflammation</td>
<td>None</td>
<td>&lt; 10 cells in cap</td>
<td>10–50 cells in cap</td>
<td>&gt; 50 cells in cap</td>
</tr>
<tr>
<td>Acute plaque inflammation</td>
<td>None</td>
<td>Occasional cells or one group &gt; 50</td>
<td>2–5 groups &gt; 50</td>
<td>&gt; 5 groups &gt; 50 or 1 group &gt; 500</td>
</tr>
<tr>
<td>Acute cap inflammation</td>
<td>None</td>
<td>&lt; 10 cells in cap</td>
<td>10–50 cells in cap</td>
<td>&gt; 50 cells in cap</td>
</tr>
<tr>
<td>Foam cells</td>
<td>None</td>
<td>&lt; 50 cells</td>
<td>&gt; 50 cells</td>
<td>–</td>
</tr>
<tr>
<td>Neovascularity</td>
<td>None</td>
<td>&lt; 10/section</td>
<td>&gt; 10/section</td>
<td>–</td>
</tr>
<tr>
<td>Cap rupture</td>
<td>Intact</td>
<td>Probably intact</td>
<td>Probably ruptured</td>
<td>Definitely ruptured</td>
</tr>
<tr>
<td>Overall Stability</td>
<td>Stable</td>
<td>Unstable</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

**Patient selection**

Recently symptomatic patients with a significant carotid artery stenosis (> 50% stenosis, based on North American Symptomatic Carotid Endarterectomy Trial [NASCET] criteria\textsuperscript{1}) who were admitted to the Leicester Vascular Surgery unit for CEA were considered for inclusion. Patients were referred by a physician specialising in stroke medicine. See Supplementary data for further details on patient selection.

**Definition of unstable plaques**

Patients were deemed to have unstable plaques based upon clinical, TCD, ultrasound, and histological evidence. Clinical criteria for instability were defined as any ischaemic event (TIA/stroke/amaurosis fugax) within the 14-day period prior to CEA. TCD criteria for instability were defined as any patient having spontaneous embolisation during preoperative monitoring or during the dissection phase of CEA prior to plaque harvest. Ultrasound criteria for plaque instability were any echolucent plaques that showed a grey-scale median (GSM) score ≤ 25. Histological instability was defined according to a modified, well-defined, well-validated American Heart Association (AHA) atherosclerotic scoring system (Table 1).\textsuperscript{14,15} Patients were grouped according to each of these criteria independently and separate analyses were performed for each definition of instability.

**Preoperative ultrasound imaging of the plaque**

Accredited ultrasonographers performed the duplex examinations using an ATL HDIS000 ultrasound scanner and an L12-5 linear array probe (Philips Medical Systems, Andover, MA, USA). GSM was measured as median of the grey values of all pixels in the plaque image.

**Surgery**

CEA was performed under general anaesthesia with TCD monitoring, and included systemic heparinisation.
(unfractionated), routine shunting, patching, and distal intimal tacking sutures.16

**Sample processing**
See online Supplementary data.

**RNA extraction from carotid plaques**
Frozen carotid atherosclerotic plaque samples were ground to a fine powder in liquid nitrogen, and total RNA was extracted using a modified Trizol (phenol-based) protocol. RNA clean up was performed using a commercially available kit (Qiagen, Hilden, Germany), which included a double DNAse step to prevent genomic DNA carry forward. The RNA integrity number (RIN) and concentration of the extracted total RNA were tested using RNA 6000 Nanodrop LabChip Kit and Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). RNA was stored at −80 °C for later analysis.

**Histopathology**
Histological specimens were analysed by two histopathologists (K.W., D.M.), who were blinded to the clinical, TCD, and ultrasound findings. For each plaque, a semi-quantitative 3- or 4-point score (Table 1)15 was assigned to show the presence and/or amount of features of plaque instability based on the AHA histological classification of advanced atherosclerotic lesions and a well-validated previously published scoring system (Figs. 1 and 2).14 Additional figures can be found in the online Supplementary Data.

**Gene expression analysis**
Microarray analysis was performed using Illumina DASL microarray (more details available in the online Supplementary data). Detailed descriptions of all data and protocols were submitted to the ArrayExpress public repository (http://www.ebi.ac.uk/miamirexpress/login.htm; accession number: E-MEXP-3683) as per MIAME guidelines.17 Genes
differentially expressed were identified by filtering, using the following criteria: (1) an Illumina diff score > 13 or < -13 (equivalent to a *p*-value < .05); and (2) a 1.3-fold difference in the mean signal between groups in each separate analysis.

Four separate analyses were performed comparing stable and unstable plaques based upon clinical (symptoms within 14 days vs. > 14 days), TCD (evidence of spontaneous embolisation vs. no detection of spontaneous embolisation), ultrasound plaque echogenicity (GSM ≤ 25 vs. > 25), and histological criteria (unstable histological classification vs. stable histological classification). The maximally differentially expressed genes from each analysis (upregulated and downregulated) were identified and the top most upregulated and single most downregulated gene from each analysis was taken forward into the validation study. Using the same samples as in the microarray experiment, differential expression was validated using quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). Only genes shown to be differentially expressed in the microarray study and subsequently validated by qRT-PCR were taken forward for replication in a larger independent cohort.

**qRT-PCR validation**

qRT-PCR was performed using TaqMan probes with all samples run in triplicate. Gene expression data were analysed by normalisation against the geometric mean of the expression of the three housekeeping genes (*B2M*, *GUSB*, and *PGK1*) showing the most stable expression from a panel.
Immunohistochemistry

Immunohistochemistry was performed on paraffin-embedded carotid plaques. Twenty-four plaques were selected from the extremes of the clinical stability/instability group (12 stable [asymptomatic] patients, 12 unstable [most recent symptom within 14 days]) and from extremes of the histologically graded plaques group (12 stable [type 0/1 plaques], 12 unstable [type 3 plaques]). Full methods available in the online Supplementary data.

Statistical analysis

Microarray study analysis was performed according to guidelines using Illumina GenomeStudio (v. 1) software. qRT-PCR-validated mRNA expression of selected genes between stable and unstable group was assessed using paired two-tailed Student t test. ELISA results were analysed using non-parametric Wilcoxon-Mann-Whitney test. ELISA results were analysed using non-parametric Wilcoxon-Mann-Whitney test.

RESULTS

Gene expression studies

The initial microarray discovery study was performed on 24 patients (Supplementary Table S1). Of these 24 patients, nine were unstable, based upon clinical criteria, nine were unstable on ultrasound criteria, six were unstable on TCD criteria, and seven were unstable on histological criteria. There were no patients with all four criteria for instability. RIN values for each of the plaques showed no significant difference when analysed according to stability group. Each plaque RNA used in the microarray study had a RIN > 6.5.

For patients with clinical instability, 177 genes were differentially expressed, with CCL19 and COX6B1 being the top up- and downregulated genes, respectively. For ultrasound instability, 1,973 genes were differentially expressed, with GAK and IGFBP7 being the top up- and downregulated genes, respectively. For TCD instability, 2,294 genes were differentially expressed, with TWF2 and SLC9A4 being the top up- and downregulated genes, respectively; and for histological instability, 134 genes were differentially expressed, with CTSG and TIMP4 being the top up- and downregulated genes, respectively. A summary table (Table 2) is shown together with heat maps (Figs. S1–S4) in

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Gene symbol</th>
<th>Analysis</th>
<th>Clinical</th>
<th>Ultrasound</th>
<th>Transcranial Doppler</th>
<th>Histology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemokine (C-C motif) ligand 19</td>
<td>CCL19</td>
<td>1.71</td>
<td>&lt; .001</td>
<td>1.37</td>
<td>&lt; .05</td>
<td>1.22</td>
</tr>
<tr>
<td>Cyclin G-associated kinase</td>
<td>GAK</td>
<td>1.35</td>
<td>&lt; .05</td>
<td>1.44</td>
<td>&lt; .05</td>
<td>1.53</td>
</tr>
<tr>
<td>Cathepsin G</td>
<td>CTSG</td>
<td>1.35</td>
<td>&lt; .05</td>
<td>1.05</td>
<td>&gt; .05</td>
<td>1.2</td>
</tr>
<tr>
<td>Signal-regulatory protein beta-1</td>
<td>SIRPB1</td>
<td>1.36</td>
<td>&lt; .05</td>
<td>1.02</td>
<td>&gt; .05</td>
<td>1.34</td>
</tr>
<tr>
<td>Insulin-like growth factor binding protein 7</td>
<td>IGFBP7</td>
<td>1.04</td>
<td>&gt; .05</td>
<td>1.37</td>
<td>&lt; .05</td>
<td>1.31</td>
</tr>
<tr>
<td>Twinfilin, actin-binding protein, homolog 2</td>
<td>TWF2</td>
<td>1.51</td>
<td>&lt; .05</td>
<td>1.35</td>
<td>&lt; .05</td>
<td>1.38</td>
</tr>
<tr>
<td>Matrix metalloproteinase-11</td>
<td>MMP11</td>
<td>1.42</td>
<td>&lt; .01</td>
<td>1.61</td>
<td>&lt; .05</td>
<td>1.13</td>
</tr>
<tr>
<td>Matrix metalloproteinase-12</td>
<td>MMP12</td>
<td>1.4</td>
<td>&lt; .05</td>
<td>1.32</td>
<td>&lt; .05</td>
<td>1.05</td>
</tr>
</tbody>
</table>

Table 2. Microarray results showing top up- and downregulated genes from each analysis.
the Supplementary data showing the top 10 up- and downregulated genes from each analysis and all of the genes found to be differentially expressed (Supplementary Tables S5 – S10).

Initial investigation sought genes that were significantly up- or downregulated in all four analyses. No genes were found to match these criteria. Eight genes were found in three out of four analyses showing concordance with direction and fold change. Table 3 shows the genes identified.

The top two genes from each group, based upon hierarchical significance and genes found to be up-/downregulated across multiple analyses, were followed up firstly by qRT-PCR validation in the same sample set to confirm the findings of the microarray study. qRT-PCR confirmed the directionality and significance of all the genes except SLC9A4 and SIRPB1.

qRT-pCR validation was then performed in an independent, larger cohort of 96 patients (for patient demographics see Supplementary Table S2) undergoing CEA. In this replication study, the significant upregulation of both CCL19 (fold change +1.89; \( p = .02 \)) and CTSG (fold change +6.05; \( p = .03 \)) was confirmed (see Table 4).

### Table 4. Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) validation of chosen genes.

<table>
<thead>
<tr>
<th>Clinical name</th>
<th>Microarray study fold change ((p))</th>
<th>qRT-PCR validation of microarray sample ((p))</th>
<th>qRT-PCR validation on independent cohort ((p))</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCL19</td>
<td>1.71 (&lt; .001)</td>
<td>1.67 (.03)</td>
<td>1.89 (.02)</td>
</tr>
<tr>
<td>COX6B1</td>
<td>−6.02 (&lt; .01)</td>
<td>−1.4 (.04)</td>
<td>1.04 (.866)</td>
</tr>
<tr>
<td>Ultrasound instability</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAK</td>
<td>1.44 (&lt; .01)</td>
<td>1.53 (.02)</td>
<td>1.2 (.48)</td>
</tr>
<tr>
<td>IGFBP7</td>
<td>−1.48 (&lt; .01)</td>
<td>−1.57 (.04)</td>
<td>2.49 (.08)</td>
</tr>
<tr>
<td>Spontaneous embolism</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SLC9A4</td>
<td>1.84 (&lt; .01)</td>
<td>1.69 (.18)</td>
<td>NA</td>
</tr>
<tr>
<td>TWF2</td>
<td>1.37 (&lt; .01)</td>
<td>1.06 (.04)</td>
<td>1.03 (.922)</td>
</tr>
<tr>
<td>Histological instability</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTSG</td>
<td>1.38 (&lt; .01)</td>
<td>2.02 (.04)</td>
<td>6.05 (.03)</td>
</tr>
<tr>
<td>TIMP4</td>
<td>−1.45 (&lt; .01)</td>
<td>−1.14 (.01)</td>
<td>−1.42 (.15)</td>
</tr>
<tr>
<td>Genes dysregulated in multiple analyses</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP11</td>
<td>1.52 (&lt; .05)</td>
<td>1.63 (.03)</td>
<td>4.6 (.06)</td>
</tr>
<tr>
<td>MMP12</td>
<td>1.41 (&lt; .05)</td>
<td>1.54 (.02)</td>
<td>1.75 (.08)</td>
</tr>
<tr>
<td>SIRPB1</td>
<td>1.42 (&lt; .01)</td>
<td>1.69 (.16)</td>
<td>NA</td>
</tr>
</tbody>
</table>

Note. NA = not applicable.

ELISA protein quantification of CCL19 and CTSG in serum

ELISA was performed on serum samples from 36 patients (\( n = 18 \) clinically unstable [most recent symptom within 7 days], \( n = 18 \) clinically stable [asymptomatic patients]) (Supplementary Table S4). Of the 36 serum samples taken from these patients, 16/36 had histologically unstable plaques.

There was a significantly higher concentration of CCL19 in the acutely symptomatic “unstable” group (median 417 pg/
mL, 95% CI 244–677 pg/mL) compared with the clinically “stable” group (median 288 pg/mL, 95% CI 138–316 pg/mL) \( (p = .02) \) (Fig. 3). Cathespin G was not identified in any serum samples, including samples taken from those patients with histologically unstable plaques.

The sensitivity values for the enzyme immunoassay were 2 pg/mL and 20 pg/mL, respectively. The intra- and inter-assay coefficients of variation were < 10% for all enzyme immunoassays.

**DISCUSSION**

This is the first study to use a whole-genome microarray technique to study the differential gene expression in unstable plaques classified according to timing from most recent event, plaque histology, plaque echogenicity, and evidence of spontaneous embolisation on TCD monitoring of the middle cerebral artery. It was found that CCL19 and CTSG were significantly upregulated in unstable plaques, and CCL19 levels were significantly higher in serum from patients who were recently symptomatic compared with those who were not. CCL19 was found to be strongly expressed around the core and cap region of recently symptomatic plaques and co-localised strongly with CD3 staining T-cell lymphocytes.

Whole-genome wide microarray has become an established and powerful research technique in the field of biomedical sciences and is approaching a quarter of a century since being first used. Since the completion of the human genome project, “whole-genome microarray” studies have given rise to the ability of surveying > 18,000 known genes simultaneously from a little over 2 μg RNA. The results of microarray studies have allowed for the search of differentially expressed genes in different patient groups or disease states and using sophisticated pathway analysis software the genetic pathways leading to observed phenotypes.

Although microarrays are an excellent tool for initial target discovery, there is a recognised variability in microarray results depending on the user, platform, and the quality of specimen (RNA) used. It is also very expensive for day-to-day diagnostic purposes and equipment is not readily available. Therefore, it is necessary to validate the results of the microarray using qRT-PCR. Results that are validated using qRT-PCR can be trusted to be accurate and minimise the risk of false-positive readings. The amount of specimen and the quality of RNA needed for qRT-PCR validation is also less than what is required for microarray, making it a potentially more accurate and accessible platform when carried forward for clinical applications.

Gene expression studies in carotid atherosclerosis using microarray have been used previously to identify differential expression between (1) symptomatic versus asymptomatic patient groups; \( ^{20–22} \) (2) postautopsy versus live tissue; \( ^{23} \) (3) cap versus intima; \( ^{24} \) (4) plaque versus normal arterial wall; \( ^{25} \) and (5) intraplaque stable and unstable regions. However, limitations of some of these studies have included small numbers used for both array work and for validation, and that, in some studies, pathway-specific arrays were examined, and therefore a whole transcriptomic approach was not used.

A wide range of biomarkers has been associated with plaque instability, including those related to different pathways: (1) proteolysis (matrix metalloproteinases 1, 2, 9, and 12); \( ^{27,28} \) (2) angiogenesis (Ang-1, Ang-2, PF4); \( ^{29,30} \) and (3) inflammation (C-reactive protein [CRP], high-sensitivity CRP, interleukin-18, transforming growth factor (TGF)-β1, pregnancy-associated plasma protein A). These have been identified within this microarray study (see Supplementary tables) but were not taken forward for further evaluation in this study as they did not meet the criteria for further investigation.

Using qRT-PCR, the microarray findings were shown to be validated with 100% concordance in directionality of fold change (up-/down-regulated) and 82% concordance with significant results. SLC9A4 and SIRPB1 both showed a similar nonsignificant trend.

Nine genes were therefore taken forward to be validated in an independent cohort. The decision to take the top most up- and downregulated genes and those genes significantly up-/downregulated across multiple array analyses was based upon avoiding selection bias that may occur if pathway or cluster analysis was used to select genes for qRT-PCR validation. In this independent, larger cohort eight out nine genes showed identical directionality of fold change, giving a concordance rate of 89%. IGFBP7 was upregulated in the larger cohort (nonsignificant), while being shown to be a downregulated gene in the microarray and initial validation study.

CCL19 (fold change 1.89; \( p = .02 \)) and CTSG (fold change 6.05; \( p = .03 \)) were shown to have significant fold changes matching the microarray and initial validation study, both being upregulated in the “unstable” larger independent cohort. These were two of the eight genes on the microarray to be upregulated in multiple analyses.

In this study, using an independent cohort of patients, it was shown that the protein expression of CCL19 and cathepsin G (protein product of CTSG) within the plaque cap and core in clinically and histologically unstable plaques. CCL19 was found to be significantly expressed in plaques from patients that had recent clinical symptoms (symptoms within the 14 days prior to surgery) and that were histologically “unstable”. CTSG was also found to be expressed more predominantly in these “unstable” plaques; however, results failed to reach significance. We demonstrated that CCL19 is co-localised with CD3-positive T-cell lymphocytes in the cap and core regions of the plaque. We also demonstrated that CD68 expression is found prominently in this region, and is reflective of macrophage activity within the plaque.

An ELISA analysis on serum from an independent cohort of patients was conducted to ascertain if CCL19 or cathepsin G were circulating proteins. The independent cohort of patients were matched for basic demographic data, with half \( (n = 18) \) having acute symptoms (symptoms within 14 days prior to surgery) and half \( (n = 18) \) being asymptomatic. Sixteen of 36 (44%) plaques from this independent...
cohort were found to be histologically unstable. This study found levels of CCL19 were significantly higher in serum from patients who were recently symptomatic compared with those who were asymptomatic. It was noted that four patients had higher than usual readings (>1,000 pg/mL) for CCL19 (one patient in the stable group and three in the unstable group). On review of the case notes, there was nothing that could account for this, so the decision was made to include these readings within the analysis.

CCL19 is a small cytokine belonging to the CC chemokine family that is also known as EBI1 ligand chemokine. The CC cytokines are proteins characterised by two adjacent cysteines and that act via G protein-coupled cell surface receptors and are expressed by lymphocytes and macrophages; they are best known for their chemotactic and proinflammatory effects. The chemokine CCL19 is a chemoattractant for different types of cells, including natural killer cells, as well as T and B lymphocytes. Macrophage inflammatory protein (MIP)-3β/CCL19 acts through CC chemokine receptor 7 (CCR7) and is a more powerful chemoattractant of lymphocytes than MIP-3α/CCL20 and other CC chemokines.

The role of CCL19 has been documented in the chemokine signalling pathway that ultimately leads to further cytokine production, cellular growth and differentiation, and apoptosis, and also influences the transendothelial migration of leukocytes. A previous study has shown a possible link between CCL19 and plaque destabilisation through recruitment of T cells and macrophages. In a recent atherogenic mouse model study, it was found that CCL19 had a significant role in cellular activation, especially of macrophages in atherosclerotic lesions. It was also noted that CCL19 influences the activation of leukocytes, lipid uptake of macrophages, and foam cell formation within atherosclerotic plaques. In a recent human coronary atherosclerotic plaque study, immunohistochemical staining of plaques showed CCL19 expression in all stages of atherosclerosis, although levels increased with the development of atherosclerosis. It was also noted that CCL19 was expressed within endothelial cells (EC) and EC dysfunction is the initiation of atherosclerotic development. This study also demonstrated higher plasma levels of CCL19 in people with coronary atherosclerotic disease, similar to the present study's findings, which showed significantly higher serum levels of CCL19 in patients who were acutely symptomatic with carotid atherosclerotic plaques.

Previous studies into human carotid artery plaques have shown T-cell and macrophage infiltration similar to atherosclerosis-prone laboratory animals. Comparison between symptomatic and asymptomatic endarterectomy specimens has revealed that plaque areas covered by inflammatory cells were significantly larger in symptomatic internal carotid artery (ICA) plaques versus asymptomatic ones, indicating that acute inflammation could also be involved in plaque destabilisation.

Acute T-cell and macrophage infiltration is most likely facilitated by an increased expression of cell adhesion molecules on the luminal surface of ICA stenoses. Interestingly, the number of dendritic cells (DC) that present antigens to T cells and thereby activate them to proliferate were also more numerous in symptomatic than asymptomatic plaques. Previous studies have linked CCL19 as a DC chemokine in patients with symptoms versus asymptomatic patients.

Proinflammatory cytokines such as IL-18 that can activate T cells and macrophages are expressed at higher levels in symptomatic ICA plaques, providing evidence that the local cytokine milieu influences plaque stability. Conversely, stable atherosclerotic ICA plaques exhibited increased expression of the anti-inflammatory cytokine TGF-β1 compared with unstable ICA plaques.

A limitation of this study is that relatively small numbers have been used in each experiment. However, this best reflects the patients recruited during the study period. It was decided that a well-conducted approach with rigorous methodology would include performing validation at each step in an independent population. In this study, this did not affect the outcome as statistically significant results were still obtained looking at CCL19 expression in both serum using ELISA and plaque using immunohistochemistry. While cathepsin G was not detected in any serum sample, suggesting this is not a circulating protein in any disease state, with larger numbers it may be proven that cathepsin G expression is statistically higher in histologically unstable plaques, as this trend was shown in this study. The limited numbers used therefore also reflect the decision to use 14 days as a cut-off for clinically symptomatic patients. Further subgroup analyses of those hyperacute (symptoms within 48 h) and those acute (symptoms 2–14 days) would depend upon larger study numbers. It was decided in this study that whole-plaque specimens were to be used in all experiments. The purpose of this was that historical gene expression studies in carotid plaques have evaluated subgroup cell types and populations, and by avoiding this targeted selection of cells the present study has avoided introducing potential bias into the analysis. In this study, owing to the heterogenous nature of human plaque specimens, it was not possible to apply multiple testing correction to microarray results; therefore, as per the manufacturer's (Illumina) recommendations, genes that had a fold change greater than or less than 1.3 were placed in hierarchical order of significance and top genes carried through for validation. However, genes that were carried through for qRT-PCR validation showed good concordance with microarray findings. A limitation of microarray studies also include potential for type II errors and missing important genes that may not have been identified as being up- or downregulated but have an important role to play in plaque instability. By using a single microarray for each sample and performing 12 microarray studies with individual and combined analyses we hope to have reduced this risk of error. Owing to the nature of tissue, there was limited tissue available after RNA extraction, so although immunohistochemical staining for proteins of interest were performed, accurate protein quantification of expressed genes could not be assessed on the tissue. Finally, it is
important to note that atherosclerosis is a systemic disease with many atherosclerotic plaques existing in the arterial system, both stable and unstable. Proinflammatory molecules may enter the circulation from any source and it is possible, that the level of CCL19 reflects the average level of plaque instability throughout the arterial system instead of being attributed to a single plaque condition. This is a limitation of any biomarker study.

Summary

In conclusion, few published studies have evaluated the role of CCL19 in acutely symptomatic carotid atherosclerotic plaques at high risk of stroke. This study has shown, through whole-genome microarray, qRT-PCR validation, ELISA, and Immunostaining, that CCL19 expression is upregulated and the level of the circulating chemokine could be used as potential biological marker of plaque instability. Its proinflammatory effects may be orchestrated through chemotaxis, cell signalling, particularly G protein-coupled receptor signalling and its involvement in the transendothelial migration of leukocytes. Future work needs to include measuring serum samples of CCL19 in a larger cohort of patients with hyperacute/crescendo symptoms and those with varied clinical presentation (stroke, TIA, amaurosis fugax). There also needs to be further work directed towards testing in an experimental model the effects of blocking expression of CCL19.

CONFLICT OF INTEREST

None.

FUNDING

This work was funded, in part, by The Circulation Foundation and The National Institute for Health Research Collaboration for Leadership in Applied Health Research and Care (NIHR CLAHRC).

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejvs.2016.05.018.

REFERENCES

3 European Carotid Surgery Trialists Collaborative Group. MRC European Carotid Surgery Trial: interim results for symptomatic patients with severe (70—99%) or mild (0—29%) carotid stenosis. Lancet 1991;337:1235—43.
21 Vemuganti R, Dempsey RJ. Carotid atherosclerotic plaques from symptomatic stroke patients share the molecular


