

# Imaging of the Vulnerable Carotid Plaque: Biological Targeting of Inflammation in Atherosclerosis using Iron Oxide Particles and MRI CME

J.M.S. Chan <sup>a</sup>, C. Monaco <sup>b</sup>, M. Wylezinska-Arridge <sup>c</sup>, J.L. Tremoleda <sup>c</sup>, R.G.J. Gibbs <sup>a,\*</sup>

<sup>a</sup> Regional Vascular Unit, St Mary's Hospital, Imperial College Healthcare NHS Trust, Imperial College London, UK

<sup>b</sup> Cytokine Biology of Atherosclerosis, Kennedy Institute of Rheumatology, Imperial College London, UK

<sup>c</sup> Biological Imaging Centre, Clinical Sciences Centre, Medical Research Council, Imperial College London, UK

## WHAT THIS PAPER ADDS

Current selection criteria for intervention in carotid disease are still determined by symptomatology and the degree of luminal stenosis. This strategy has not been effective in identifying a high-risk asymptomatic subgroup, and much effort has been directed at identifying asymptomatic but vulnerable plaques. This work describes the development of a dual targeting strategy directly reporting arterial endothelial activation in human plaque tissue by using antibody-conjugated SPIO particles as MRI probes for inflammation. These MRI probes could potentially provide clinicians with a novel imaging tool for characterization of atherosclerosis at a molecular level, thereby permitting accurate risk stratification in carotid disease.

**Objectives:** Identification of those patients with high-risk asymptomatic carotid plaques remains an elusive but essential step in stroke prevention. Inflammation is a key process in plaque destabilization and the propensity of atherosclerotic lesions to cause clinical sequelae. There is currently no clinical imaging technique available to assess the degree of inflammation associated with plaques. This study aims at visualizing and characterizing atherosclerosis using antibody-conjugated superparamagnetic iron oxide (SPIO) particles as an MRI probe to assess inflammation in human atherosclerotic plaques.

**Methods:** Atherosclerotic plaques were collected from 20 consecutive patients ( $n = 10$  from symptomatic patients,  $n = 10$  from asymptomatic patients) undergoing carotid endarterectomy (CEA) for extracranial high-grade internal carotid artery (ICA) stenosis ( $>70\%$  luminal narrowing). Inflammatory markers on human atherosclerotic plaques were detected and characterized by ex vivo magnetic resonance imaging (MRI) using anti-VCAM-1 antibody and anti-E-selectin antibody-conjugated SPIO with confirmatory immunohistochemistry.

**Results:** Inflammation associated with human ex vivo atherosclerotic plaques could be imaged using dual antibody-conjugated SPIO by MRI. Symptomatic plaques could be distinguished from asymptomatic ones by the degree of inflammation, and the MR contrast effect was significantly correlated with the degree of plaque inflammation ( $r = .64$ ,  $p < .001$ ). The asymptomatic plaque population exhibited heterogeneity in terms of inflammation. The dual-targeted SPIO-induced MR signal not only tracked closely with endothelial activation (i.e. endothelial expression of VCAM-1 and E-selectin), but also reflected the macrophage burden within plaque lesions, offering a potential imaging tool for quantitative MRI of inflammatory activity in atherosclerosis.

**Conclusions:** These functional molecular MRI probes constitute a novel imaging tool for ex vivo characterization of atherosclerosis at a molecular level. Further development and translation into the clinical arena will facilitate more accurate risk stratification in carotid artery disease in the future.

© 2014 European Society for Vascular Surgery. Published by Elsevier Ltd. All rights reserved.

Article history: Received 4 October 2013, Accepted 21 January 2014, Available online 1 March 2014

**Keywords:** Vulnerable plaque, Magnetic resonance imaging, Iron oxide particles, Inflammation, Atherosclerosis

## INTRODUCTION

Carotid endarterectomy (CEA) for symptomatic internal carotid artery (ICA) atherosclerotic lesions is a well-recognized intervention in stroke prevention. The case for intervention in asymptomatic lesions has become more controversial with the development of better medical preventative treatment, and with an annual stroke risk of 1–2%<sup>1</sup> for asymptomatic threshold disease it is more difficult to justify surgery or stenting. Nonetheless, asymptomatic benign atherosclerotic lesions do go on to become unstable

**CME** To access continuing medical education questions on this paper, please go to [www.vasculareducation.com](http://www.vasculareducation.com) and click on 'CME'

\* Corresponding author. R.G.J. Gibbs, Regional Vascular Unit, St Mary's Hospital, Imperial College Healthcare NHS Trust, Imperial College London, UK.

E-mail address: [r.gibbs@imperial.ac.uk](mailto:r.gibbs@imperial.ac.uk) (R.G.J. Gibbs).

1078-5884/\$ — see front matter © 2014 European Society for Vascular Surgery. Published by Elsevier Ltd. All rights reserved.

<http://dx.doi.org/10.1016/j.ejvs.2014.01.017>

and lead to thromboembolic stroke, but there is no current reliable methodology to identify these lesions. Promising non-invasive imaging techniques, such as contrast-enhanced ultrasound, high-resolution magnetic resonance imaging (MRI), and co-registered positron emission tomography with computed tomography (CT) and MRI are currently in development in order to interrogate plaque vulnerability *in vivo*. High-resolution MRI has emerged as a leading non-invasive imaging modality for assessing atherosclerosis, because of its excellent spatial resolution at a submillimetre level, soft-tissue contrast, and high signal-to-noise ratio. The significant advantage of MRI is the ability to acquire and combine multiple different contrast weightings to characterize tissue composition within the vessel wall, resulting in the quantification of the main plaque components such as intraplaque haemorrhage, lipid-rich necrotic core, calcification, and surface disruption.<sup>2</sup> There are two types of MRI contrast agents, namely paramagnetic and superparamagnetic, which increase sensitivity and facilitate diagnosis by shortening T1 and T2 relaxation times respectively. In contrast to paramagnetic contrast agents (gadolinium) that create hyperintense signals (“positive” contrast) in T1-weighted images, superparamagnetic contrast agents shorten the transverse T2 and T2\* relaxation times, resulting in hypointense signals that appear black on T2- and T2\*-weighted MR images (“negative” contrast).<sup>3</sup> These superparamagnetic agents are based on iron oxide particles, which are composed of a core of iron oxides, coated by a biocompatible inert polymer, usually dextran.<sup>4</sup>

However, to fully capture the diagnostic potential of MRI requires imaging at molecular and cellular levels. Molecular MRI comprises “passive” and “active” imaging strategies. Ultrasmall superparamagnetic particles of iron oxide (USPIO) have been used as passive contrast agents to identify plaque macrophages as surrogate markers of plaque inflammation in the assessment of atherosclerosis in human and animal models.<sup>5–7</sup> “Active” molecular imaging involves direct reporting of specific molecular events, which mandates the use of a specific targeting system. Target specificity of contrast agents can be customized through conjugation of a variety of targeting ligands, such as monoclonal antibodies, antibody fragments (Fab), peptides or sugars, to functional groups on the surface of iron oxide particles.<sup>8,9</sup>

Inflammation is a key driver of plaque instability. Macrophages play a critical role in promoting fibrous cap degradation and plaque destabilization, converting chronic stable atherosclerotic lesions into acute unstable lesions with the potential for thromboembolism. Monocyte recruitment into vascular tissues is promoted by the overexpression of adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1; CD106), E-selectin (CD62E), and P-selectin (CD62P), on the activated endothelium.<sup>10</sup> The monocyte–endothelial binding mechanism represents an opportunity to exploit both cell adhesion molecules and selectins on the activated endothelium as targets to directly report arterial endothelial activation and inflammation. A dual targeting strategy directed at E-selectin and VCAM-1 using antibody-conjugated superparamagnetic particles of iron oxide

(SPIO) to act as a contrast agent in order to render activated arterial endothelium MR visible has been developed.

In this article the detection and characterization of E-selectin and VCAM-1 on excised human carotid plaques using antibody-conjugated SPIO and immunohistochemistry is described. The extent to which antibody-conjugated SPIO-induced MR signal tracks endothelial activation across a range of atherosclerotic lesion complexities in humans by *ex vivo* MRI is evaluated. It was hypothesized that symptomatic carotid plaques could be discriminated from asymptomatic ones predicated on the degree of inflammation detected, and additionally sought to determine whether asymptomatic plaques could be distinguished based on the degree of inflammation exhibited using these MRI probes.

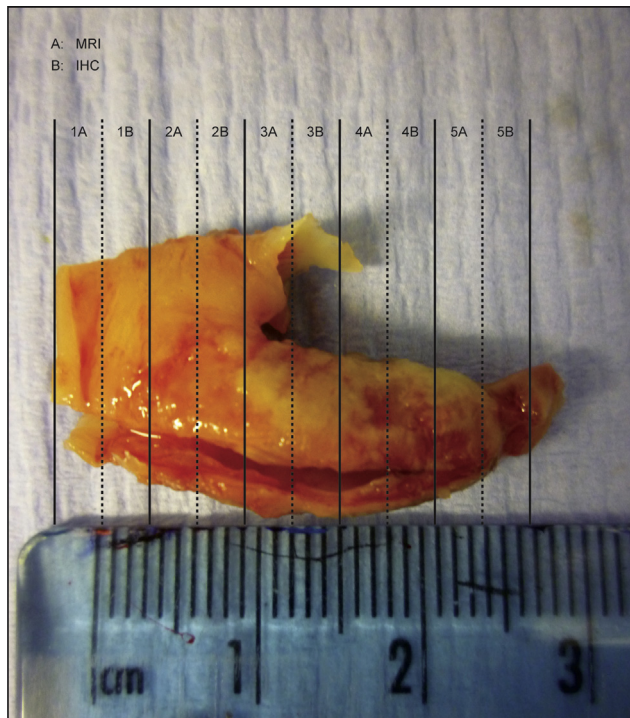
## MATERIALS AND METHODS

### Study population

Atherosclerotic plaques were collected from 20 consecutive patients ( $n = 10$  from symptomatic patients,  $n = 10$  from asymptomatic patients) undergoing CEA for extracranial high-grade ICA stenosis of 70% or greater, using the North American Symptomatic Carotid Endarterectomy Trial criteria. Symptomatic patients were defined as those who had a history of recent (less than 4 months before enrolment) transient retinal or cerebral symptoms or ischaemic stroke attributable to the high-grade ICA lesion.<sup>11</sup> Asymptomatic patients were defined as those who had no history or only remote (more than 4 months) ischaemic symptoms. The symptomatic group had presented with cerebrovascular transient ischaemic attacks ( $n = 8$ ) or ischaemic stroke ( $n = 2$ ) within the preceding 4 months. All other specimens were collected from 10 clinically asymptomatic patients exhibiting ipsilateral ICA stenosis of >70% on serial ultrasound duplex. The degree of ICA stenosis was established in two consecutive ultrasound duplex before surgery in all cases, and additionally confirmed by either CT angiography (CTA) or MR angiography (MRA). Preoperatively all patients underwent neurological evaluation. Macroscopically normal specimens adjacent to endarterectomized plaques were harvested from patients ( $n = 2$ ) undergoing femoral endarterectomy to serve as controls for MRI and histology. The study was conducted from 2009 to 2012 and was approved by the local ethics committee. Written informed consent was obtained from all patients.

### Tissue collection

All patients underwent CEA in the regional vascular unit at St Mary's Hospital, London, UK. The median duration of patients undergoing CEA after symptom onset was 9 (range 5–35) days. At operation the carotid artery bifurcation was endarterectomized with the intention to remove the whole plaque intact. The excised plaque sample consisted of intima and inner medial layers of the artery. Thereafter the plaque was rinsed with phosphate-buffered saline (PBS) and cut into five sections, each of which was further subdivided into two smaller adjacent sections (Fig. 1). Great care was taken to prevent plaque disintegration during division of the



**Figure 1.** Carotid endarterectomy (CEA) specimen cut into sections. At CEA operation, the carotid artery bifurcation was endarterectomized with the aim of removing the whole plaque intact. The plaque was cut into five sections, each of which was further subdivided into two smaller adjacent sections. One adjacent section was used for magnetic resonance imaging detection and characterization of inflammatory markers, whilst the other adjacent section was used for immunohistochemistry.

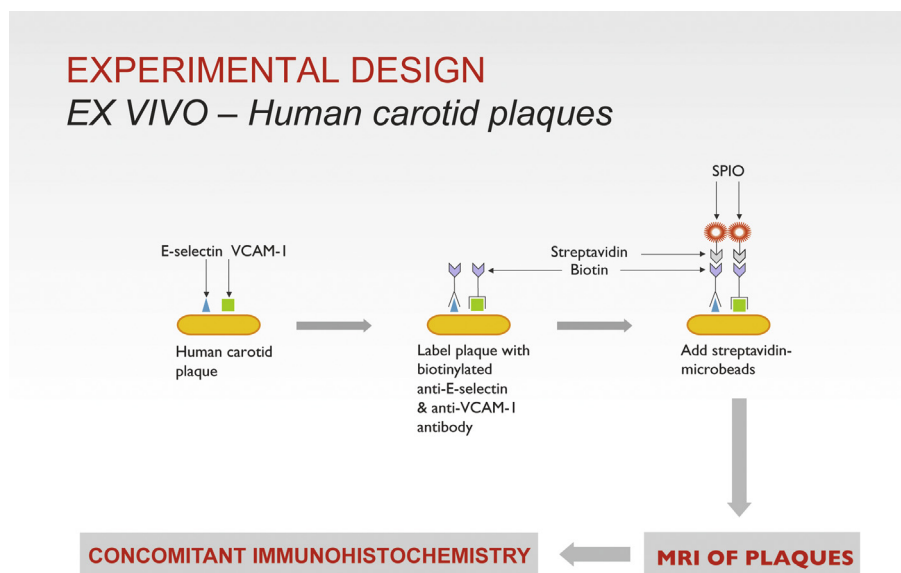
unfixed specimens. The orientation and sequence of the sections were marked and recorded. One adjacent section was fixed in 4% paraformaldehyde (PFA) at 4 °C for MRI detection and characterization of inflammatory markers, while the other adjacent section was fixed in formalin and embedded in paraffin blocks for immunohistochemistry. Concomitant immunohistochemistry was performed to correlate MRI data.

### **Detection, characterization, and imaging of inflammatory markers on human carotid plaques by biotinylated antibody-conjugated streptavidin microbeads**

Following fixation in 4% PFA, plaque sections were rinsed in PBS. They were incubated with 2% horse serum at room temperature for 1 hour to block non-specific binding of antibodies. Plaque sections were then incubated with biotinylated mouse anti-human E-selectin monoclonal antibody (dilution 10 µg/mL, R&D Systems, Abingdon, UK) and biotinylated mouse anti-human VCAM-1 monoclonal antibody (dilution 10 µg/mL, GenWay Biotech, San Diego, USA) for 20 minutes at room temperature. Plaque sections were washed twice to remove unbound biotinylated antibodies by incubating with labelling buffer composed of PBS, supplemented with 2 mM EDTA at 4 °C for 10 minutes. To make the plaques become “MR visible”, they were incubated in streptavidin microbeads (Miltenyi Biotec Ltd, Surrey, UK), that is magnetic beads (50 nm diameter SPIO) conjugated to streptavidin, diluted in labelling buffer (dilution 1:10) at 4 °C for 15 minutes (Fig. 2).

### **Ex vivo MRI of human carotid plaques**

The carotid plaque from each patient was divided into five smaller sections giving symptomatic ( $n = 50$ ), asymptomatic



**Figure 2.** Experimental design of ex vivo human plaque stage. The inflammatory markers on human carotid plaque were detected by biotinylated antibodies, which were in turn bound to the streptavidin microbeads, thereby making these plaques “MR visible”. Concomitant immunohistochemistry was performed.



**Figure 3.** Human carotid plaque sections embedded in agarose block. A tube of microbeads was also embedded in the agarose block to act as positive control.

( $n = 50$ ), control femoral artery sections ( $n = 10$ ), together with three controls (symptomatic carotid plaque incubated with biotinylated non-immune IgG antibodies and microbeads, symptomatic carotid plaque, and streptavidin microbeads). All were embedded in 2% agarose (Fig. 3). Ex vivo MRI of human carotid plaques was performed using a 9.4-Tesla, horizontal bore scanner (Varian Inc., Palo Alto, CA, USA) running VnmrJ 2.3A software. The sample was placed in a quadrature birdcage coil (40 mm internal diameter). T2 spin echo sequence parameters are listed in the [Supplementary Material](#).

#### Quantitative MR image analysis

MR images were analysed by ImageJ 1.43r software (National Institutes of Health, <http://rsb.info.nih.gov/ij/index.html>). The contours of each plaque section on the MR image were delineated manually and independently by two observers and defined as the region of interest (ROI). The signal within the ROI (plaque signal) was measured using ImageJ 1.43r software. The contrast effect in each plaque section was quantified by T2 value, which was calculated using GraphPad Prism 5 software (GraphPad software Inc., San Diego, USA).

#### Microscopy and quantification of immunostaining

Wide-field microscopy was performed with a Zeiss Axiovert 200 inverted microscope. The area of positive staining of VCAM-1, E-selectin, CD68 (macrophages), CD31 (endothelial cells), and Perls' stain (intralesional iron) in each plaque section was objectively quantified using Volocity 5.5 image analysis software (PerkinElmer, Boston, MA, USA). The stained area of Perls' (iron) and CD68 (macrophages) was quantified as a percentage of the total lesion area.<sup>12</sup> CD31 is commonly used to demonstrate the presence of endothelial cells. Hence the area of CD31 expression was considered as

a total area of intact endothelium. Quantitative stereological analysis of VCAM-1 and E-selectin endothelial expression was related to CD31 staining in endothelium and calculated as

$$\text{VCAM-1/E-selectin expression on endothelial cells (\%)} = \frac{[\text{Area (x)}/\text{Area (CD31)}] \times 100\%}{\text{where area (x) is the area of VCAM-1 or E-selectin expression in the endothelium and area (CD31) is the area of CD31 expression in the endothelium.}^{13}}$$

*Details concerning immunohistochemistry, intra/interobserver variability in the quantification of the contrast effect are found in the [Supplementary Data](#)*

**Statistical methods.** To assess differences in categorical variables in patient clinical characteristics, the Fisher exact test (two-tailed) was used. The relationship between the mean macrophage content and MR contrast effect quantified by mean  $1/T_2$  of the whole plaque per patient was assessed by Spearman's correlation. Further, the whole plaque has signal change assessed in five smaller plaque sections in order to minimize the chance of a focal signal change being "lost" in a mean signal change for the whole plaque. However, each of these plaque sections cannot be examined as an independent observation as the signal in one section will affect that in its adjacent section. To reduce potential statistical error, a complex repeated measures modelling was chosen to represent true variances in section data, thereby yielding the degrees of freedom to the correct level. The strength of the relationship between histological marker data (expression of VCAM-1, E-selectin, CD68 and Perls' iron stain) and MR image data in each of the five carotid plaque sections per patient was assessed using correlation and regression analysis. Since data were collected on five sections per patient, repeated measures regression model were fitted by using MR image data as

response variables and histological marker data as predictor variables, taking the symptom status of patient into account.

Repeated measures analysis of variance (ANOVA) was performed using general linear model function in Statistical Analysis System 9.1 software (SAS Institute, Inc, Cary, NC, USA). A value of  $p < .05$  was considered statistically significant. Intraobserver and interobserver variability in T2 values were analysed using a Bland–Altman plot.

## RESULTS

### Baseline demographics

Baseline demographics and clinical risk factors were similar in both symptomatic and asymptomatic patient groups (Table 1).

### Relationship between plaque macrophage content and MR contrast effect

This study showed that the degree of inflammation associated with human atherosclerotic plaques could be imaged using dual antibody-conjugated SPIO by ex vivo MRI. Fig. 4 demonstrates a spectrum of MR images ranging from phenotypically symptomatic inflamed plaques, appearing darkest, followed by the asymptomatic plaques, to control femoral artery and negative controls (symptomatic plaque only and symptomatic plaque with IgG and microbeads), appearing brightest. Macrophage content in plaque was used as an index of plaque inflammation. Plaques were

defined as inflamed if there was greater than 5% of macrophage staining in the total lesion area. Importantly, it was possible to observe a degree of heterogeneity in the asymptomatic plaque population based on the varying degree of inflammation, with inflamed plaques appearing darker than non-inflamed ones. Consistent with the MR images, the concomitant immunohistochemistry for CD68 (macrophages) was observed to be the most abundant on phenotypically symptomatic inflamed plaques, followed by the inflamed asymptomatic ones, then the non-inflamed asymptomatic ones and the least for the control femoral artery sections.

To quantify these observations, the mean 1/T2 value (MR contrast effect) of whole plaque was calculated. Fig. 5 demonstrates that the mean macrophage content (index of plaque inflammation) is significantly correlated with the mean 1/T2 of the whole plaque per patient ( $r = .64$ ,  $p < .001$ ). Consistent with the MRI results, the symptomatic plaques (in red) with the greater level of inflammation produced a higher 1/T2 value and appeared darker, followed by the inflamed asymptomatic plaques (in orange), then the non-inflamed plaques (in yellow).

### Relationship between expression of histological markers and MR contrast effect

Taking the symptom status of each patient into account, the strength of the relationship between histological marker data (expression of VCAM-1, E-selectin, CD68, and Perls' iron stain) and the MR contrast effect in each of the five carotid plaque sections per patient was assessed. The fitted regression model with endothelial expression of VCAM-1 in carotid plaque sections as predictors and 1/T2 as response variable controlling for symptom status of patient reported  $R^2 = .79$ , indicating that 79% of the variation in 1/T2 is fitted by the endothelial VCAM-1 expression in the plaque sections. The overall regression model was found to be statistically significant,  $F(9, 90) = 37.93$ ,  $p < .01$ . The  $t$  test demonstrated that the estimated regression model parameter for endothelial VCAM-1 expression was found to be statistically significant ( $b = 0.00224$ ,  $t(1) = 7.317$ ,  $p < .001$ ). Therefore endothelial VCAM-1 expression in carotid plaque sections and 1/T2 is significantly strongly correlated. Similarly, endothelial expression of E-selectin in carotid plaque sections and 1/T2 was also significantly strongly correlated ( $R^2 = 0.82$ ,  $F(9, 90) = 46.71$ ,  $p < .01$ ), ( $b = 0.00339$ ,  $t(1) = 7.48$ ,  $p < .001$ ). In comparison, CD68 expression in carotid plaque sections and 1/T2 was statistically significant, yet moderately correlated ( $R^2 = 0.5687$ ,  $F(4, 105) = 34.61$ ,  $p < .001$ ;  $b = 0.001$ ,  $t(1) = 5.13$ ,  $p < .0001$ ). The effect of intraplaque iron content, indicative of past intraplaque haemorrhage, as visualized by Perls' stain in carotid plaque sections on 1/T2 was not statistically significant.

### Synergistic relationship of E-selectin and VCAM-1 expression on 1/T2

Repeated measures ANOVA was performed to test the significance of the effect of VCAM-1 and E-selectin

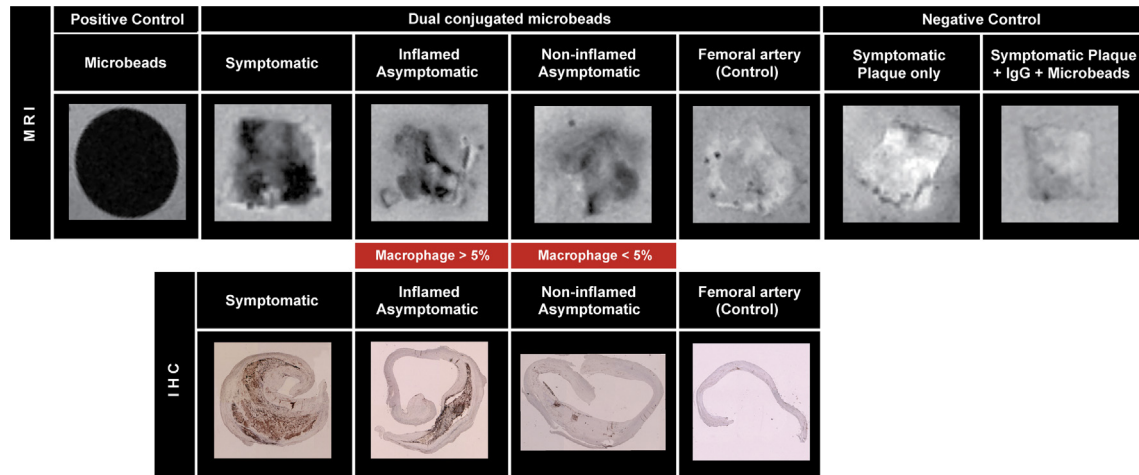
**Table 1.** Patient clinical characteristics.

Variable	Symptomatic	Asymptomatic	$p$
Median age (range)	73 (67–76)	72 (66–77)	0.7039 <sup>b</sup>
Gender, male/female	7/3	6/4	1.0000
TIA, no.	8	0	0.0007 <sup>a</sup>
Stroke, no.	2	0	0.4737
Ischaemic heart disease, no.	4	5	1.0000
Diabetes mellitus, no.	2	3	1.0000
Hypertension, no.	7	7	1.0000
Hypercholesterolaemia, no.	7	8	1.0000
Smoking, no.	4	6	0.6563
Medications: statin, no.	6	8	0.6285
Medications: antihypertensives, no.	7	7	1.0000
Medications: aspirin, no.	5	7	0.6499
Medications: Clopidogrel, no.	4	0	0.0867
Medications: NSAIDs, no.	2	1	1.0000

Note. NSAIDs, non-steroidal anti-inflammatory drugs; TIA, transient ischaemic attack.

<sup>a</sup> None of these characteristics differ significantly other than that for TIA, as determined by the Fisher exact test.

<sup>b</sup> No significant difference in age was found between the two patient groups, as determined by the Mann-Whitney U test.



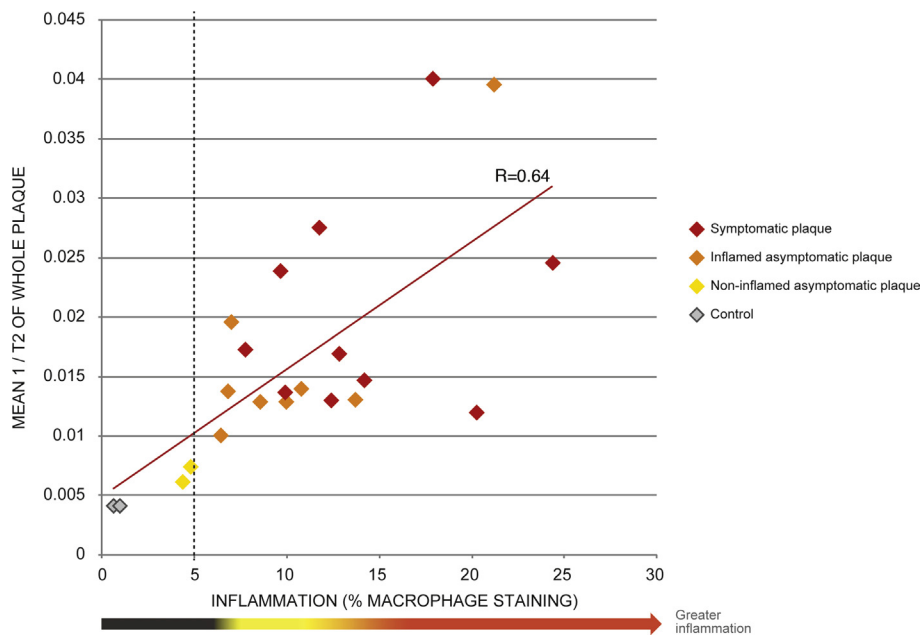
**Figure 4.** Spectrum of magnetic resonance (MR) images and histological images of phenotypically symptomatic plaques, asymptomatic plaques and control femoral artery. The spectrum of MR images in the upper row range from phenotypically symptomatic inflamed plaques, appearing darkest, followed by the asymptomatic plaques, to control femoral artery and negative controls (symptomatic plaque only and symptomatic plaque with IgG and microbeads), appearing brightest. Importantly, a degree of heterogeneity in the asymptomatic plaque population is detected, with inflamed plaques appearing darker than non-inflamed ones. Consistent with the MR images, the concomitant immunohistochemistry for CD68 (macrophages as indicated by brown stain) in the lower row is observed to be the most abundant on phenotypically symptomatic inflamed plaques, followed by the inflamed asymptomatic ones, then the non-inflamed asymptomatic ones and the least for the control femoral artery sections.

endothelial expression and their interaction on 1/T2 in each of the five carotid plaque sections per patient. VCAM-1 expression and E-selectin expression explained 94.08% of the variation in 1/T2 in carotid plaque sections ( $R^2 = 0.9408$ ) and was statistically significant ( $F(1, 103) = 272.64, p < .001; b = 0.00015, t(1) = 8.96, p < .01$ ). Compared with using E-selectin or VCAM-1 expression alone, there was an increase of 12% and 15% respectively in the explanatory power by using E-selectin

and VCAM-1 expression together. Therefore it is concluded that there is a significant synergistic relationship between VCAM-1 and E-selectin endothelial expression on 1/T2.

**DISCUSSION**

A biological dual-targeting contrast strategy directly reporting the arterial endothelial activation attracting the monocytes in human plaque tissue has been developed in



**Figure 5.** The relationship between intraplaque inflammation and magnetic resonance (MR) contrast effect. The mean macrophage content (index of plaque inflammation) is significantly correlated with the mean 1/T2 (MR contrast effect) of the whole plaque per patient ( $r = .64, p < .001$ ). Consistent with the MRI results, the symptomatic plaques (in red) with the greater level of inflammation produce a higher 1/T2 value and appear darker; followed by the inflamed asymptomatic plaques (in orange), then the non-inflamed plaques (in yellow).

order to interrogate plaque vulnerability and add further prognostic information to luminal stenosis alone. It has been possible to discriminate symptomatic plaques from asymptomatic ones predicated on the degree of inflammation, and, more importantly, inflamed plaques from the non-inflamed ones within the asymptomatic plaque population by ex vivo MRI. This study showed that the mean  $1/T_2$  value of the whole plaque was significantly correlated with plaque inflammation. Supported by previous post-mortem and atherectomy studies, increased expression of VCAM-1, E-selectin, and P-selectin was known to correlate with a high density of macrophages and T lymphocytes in the inflamed coronary and carotid plaques.<sup>14</sup> These data suggest that higher levels of VCAM-1 and E-selectin endothelial expression from the inflamed plaques resulted in greater binding of antibody-conjugated microbeads to the endothelial cells, producing a greater signal loss in T2 spin echo sequence and greater  $1/T_2$  value.

This was confirmed by further analysis using repeated measures modelling to assess the strength of the relationship between histological marker data and MR contrast effect in each of the five carotid plaque sections per patient. As previously described,<sup>15</sup> this complex statistical model aims to provide a more robust analysis of the data than simple paired analysis. In this model, a stronger correlation between endothelial expression of VCAM-1 and E-selectin with  $1/T_2$  was demonstrated than that of CD68 with  $1/T_2$ , suggesting that the antibody-conjugated iron particles predominantly targeted activated endothelial cells rather than macrophages. The dual-targeted SPIO-induced MR signal not only tracked closely with endothelial activation but unsurprisingly also reflected the macrophage burden within plaque lesion, suggesting that the degree of inflammation and endothelial activation correlated with the number of plaque-based macrophages. The results were consistent with the role of increased endothelial expression of adhesion molecules in promoting monocyte recruitment into vascular tissues.<sup>10,16</sup> The present results affirm that the MRI combined with the dual-targeted SPIO approach can potentially offer a novel imaging tool for quantitative assessment of inflammation across a range of atherosclerotic lesions complexities in the future.

Furthermore, this study has utilized selectin-mediated monocyte rolling and VCAM-1-mediated firm adhesion mechanisms to develop the antibody-conjugated SPIO. This work demonstrated a significant synergistic relationship between VCAM-1 and E-selectin endothelial expression on  $1/T_2$  value, supporting a synergistically augmented binding of dual antibody-conjugated iron particles to atherosclerosis, compared with either in isolation. Although this is ex vivo work, it is the first study to demonstrate the synergistically augmented binding effect of the dual antibody-conjugated iron particles on human atherosclerotic plaques.

#### Limitations and future work

The very high binding affinity of biotin with streptavidin has led to the use of this binding complex in a wide variety of

in vitro and in vivo applications. However, the significant level of endogenous biotin present in serum and the slow dissociation of the biotin–streptavidin complex can pose a potential limitation of the translation of biotinylated antibody-conjugated streptavidin microbeads in clinical diagnostic and therapeutic applications. Moreover, the size of the SPIO was 50 nm in diameter. Compared with microparticles of iron oxide, SPIO can only convey a relatively small payload of iron, limiting its effect on local magnetic field homogeneity, and therefore compromising the contrast sensitivity. The substantial contrast effect and sensitivity of the dual antibody-conjugated SPIO demonstrated in this ex vivo study could be undermined when being translated to the in vivo condition. The small particle size permits SPIO to enter atherosclerotic plaques, potentially compromising the specificity of these particles for molecular targets expressed on vascular endothelium. However, the short incubation period of SPIO with plaque sections limits their passive diffusion into plaques. This was supported by the minimal signal loss on the negative control, symptomatic plaque incubated with non-immune IgG and microbeads, suggesting that non-specific binding or diffusion of SPIO to plaque sections was minimal (Fig. 4).

Future work will use iron oxide particles of larger size in order to enhance their sensitivity to achieve in vivo detection of low-abundance endothelial molecular targets. The streptavidin–biotin link between the biotinylated primary antibody and streptavidin microbeads will need to be replaced by a direct conjugation of antibodies to the iron oxide particles to bring the methodology a step closer to a practical clinical application.

#### CONCLUSION

Using the dual antibody-conjugated SPIO, the degree of inflammation associated with human atherosclerotic plaques has been imaged by ex vivo MRI. More importantly, the potentially high-risk inflamed plaques have been identified from the non-inflamed ones within the asymptomatic plaque population. The dual-targeted SPIO-induced MR signal not only tracked closely with endothelial activation (i.e. endothelial expression of VCAM-1 and E-selectin), but also reflected the macrophage burden within plaque lesions, offering a potential imaging tool for quantitative MRI of inflammatory activity in atherosclerosis. Moreover, this study has demonstrated the synergistically augmented binding effect of the dual antibody-conjugated SPIO on human atherosclerotic plaques. These functional molecular MRI probes potentially provide clinicians with a novel imaging tool for in vivo characterization of atherosclerosis at a molecular level, thereby permitting more accurate risk stratification in carotid artery disease and optimizing resource allocation to target the high-risk cohorts in the future.

#### FUNDING

This research was funded by The European Society for Vascular Surgery, The St Mary's Trustees Charitable Fund and The Royal College of Surgeons of England.

**CONFLICT OF INTEREST**

None.

**APPENDIX A. SUPPLEMENTARY DATA**

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.ejvs.2014.01.017>.

**REFERENCES**

- 1 Nicolaides AN, Kakkos SK, Griffin M, Sabetai M, Dhanjil S, Tegos T, et al. Severity of asymptomatic carotid stenosis and risk of ipsilateral hemispheric ischaemic events: results from the ACSRS study. *Eur J Vasc Endovasc Surg* 2005;**30**: 275–84.
- 2 Saam T, Ferguson MS, Yarnykh VL, Takaya N, Xu D, Polissar N, et al. Quantitative evaluation of carotid plaque composition by in vivo MRI. *Arterioscler Thromb Vasc Biol* 2005;**25**:234–9.
- 3 Rudd JH, Hyafil F, Fayad ZA. Inflammation imaging in atherosclerosis. *Arterioscler Thromb Vasc Biol* 2009;**29**:1009–16.
- 4 Tang TY, Muller KH, Graves MJ, Li ZY, Walsh SR, Young V, et al. Iron oxide particles for atheroma imaging. *Arterioscler Thromb Vasc Biol* 2009;**29**(7):1001–8.
- 5 Kooi ME, Cappendijk VC, Cleutjens KB, Kessels G, Kitslaar PJ, Borgers M, et al. Accumulation of ultrasmall superparamagnetic particles of iron oxide in human atherosclerotic plaques can be detected by in vivo magnetic resonance imaging. *Circulation* 2003;**107**:2453–8.
- 6 Trivedi RA, U-King-Im J-M, Graves MJ, Cross JJ, Horsley J, Goddard MJ, et al. In vivo detection of macrophages in human carotid atheroma: temporal dependence of ultrasmall superparamagnetic particles of iron oxide-enhanced MRI. *Stroke* 2004;**35**:1631–5.
- 7 Sigovan M, Bousset L, Sulaiman A, Sappey-Mariniere D, Alsaïd H, Desbleds-Mansard C, et al. Rapid-clearance iron nanoparticles for inflammation imaging of atherosclerotic plaque: initial experience in animal model. *Radiology* 2009;**252**:401–9.
- 8 McAteer MA, Akhtar AM, von Zur Muhlen C, Choudhury RP. An approach to molecular imaging of atherosclerosis, thrombosis, and vascular inflammation using microparticles of iron oxide. *Atherosclerosis* 2010;**209**:18–27.
- 9 Choudhury RP, Fisher EA. Molecular imaging in atherosclerosis, thrombosis, and vascular inflammation. *Arterioscler Thromb Vasc Biol* 2009;**29**(7):983–91.
- 10 Davies MJ, Gordon JL, Gearing AJ, Pigott R, Woolf N, Katz D, et al. The expression of the adhesion molecules ICAM-1, VCAM-1, PECAM, and E-selectin in human atherosclerosis. *J Pathol* 1993;**171**:223–9.
- 11 North American Symptomatic Carotid Endarterectomy Trial (NASCET) Steering Committee. North American Symptomatic Carotid Endarterectomy Trial: methods, patient characteristics, and progress. *Stroke* 1991;**22**:711–20.
- 12 Jander S, Sitzer M, Schumann R, Schroeter M, Siebler M, Steinmetz H, et al. Inflammation in high-grade carotid stenosis: a possible role for macrophages and T cells in plaque destabilization. *Stroke* 1998;**29**:1625–30.
- 13 Nachtigal P, Jamborova G, Pospisilova N, Pospisilova K, Solichova D, Zdansky D, et al. Atorvastatin has distinct effects on endothelial markers in different mouse models of atherosclerosis. *J Pharm Pharm Sci* 2006;**9**:222–30.
- 14 O'Brien KD, McDonald TO, Chait AA, Allen MD, Alpers CE. Neovascular expression of E-selectin, intercellular adhesion molecule-1, and vascular cell adhesion molecule-1 in human atherosclerosis and their relation to intimal leukocyte content. *Circulation* 1996;**93**:672–82.
- 15 Tang TY, Howarth SP, Li ZY, Miller SR, Graves MJ, U-King-Im JM, et al. Correlation of carotid atheromatous plaque inflammation with biomechanical stress: utility of USPIO enhanced MR imaging and finite element analysis. *Atherosclerosis* 2008;**196**: 879–87.
- 16 McAteer MA, Mankia K, Ruparelina N, Jefferson A, Nugent N, Stork LA, et al. A leukocyte-mimetic magnetic resonance imaging contrast agent homes rapidly to activated endothelium and tracks with atherosclerotic lesion macrophage content. *Arterioscler Thromb Vasc Biol* 2012;**32**:1427–35.