Noninvasive Assessment of Atherosclerotic Plaque Progression in ApoE\(^{-/-}\) Mice Using Susceptibility Gradient Mapping

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**Background**—Macrophages have been identified as a major contributor to plaque development and destabilization in atherosclerosis. The aim of this study was to noninvasively assess uptake of citrate coated very small iron oxide particles at different stages of plaque development in the brachiocephalic artery of apoE\(^{-/-}\) mice. Susceptibility gradient mapping (SGM) was applied to generate positive contrast images and to quantify iron oxide uptake.

**Methods and Results**—ApoE\(^{-/-}\) mice were fed a high-fat diet for 4, 8, or 12 weeks; 300 \(\mu\)mol Fe/kg was injected 24 and 48 hours before final MRI. Increasing very small iron oxide particle uptake was observed over the course of atherosclerotic plaque development. Simultaneous administration of pravastatin led to a significant decrease in very small iron oxide particle uptake, assessed by mass spectroscopy and histology. SGM-MRI allowed the generation of positive contrast images, and magnitudes (mT/m) of contrast enhancement in SG parameter maps significantly correlated with the absolute iron oxide content (\(R^2=0.70, P<0.05\)) and the macrophage density (\(R^2=0.71, P<0.05\)).

**Conclusions**—This study shows an increase in iron oxide uptake (measured by in vivo SGM-MRI, histology, and mass spectroscopy) with the progression of plaque development in an apoE\(^{-/-}\) mouse model of accelerated atherosclerosis. Positive contrast provided by SGM-MRI allowed for a clear visualization of intraplaque iron oxide depositions, and magnitudes (mT/m) of contrast enhancement in SG parameter maps allowed for the quantification of intraplaque iron oxide particles. (Circ Cardiovasc Imaging. 2011;4:295-303.)

**Key Words:** atherosclerosis • molecular imaging • MRI • plaque • iron oxide

Coronary artery disease remains the leading cause of death in the Western world and developing countries, with the majority of complications resulting from plaque rupture and subsequent thrombosis.\(^1\) At present, invasive x-ray coronary angiography is the method of choice in clinical practice for the detection of obstructive coronary artery disease. Although x-ray angiography allows the assessment of luminal narrowing, it provides little information on plaque composition and biology, resulting in the inability to clearly distinguish between stable and unstable lesions.

**Clinical Perspective on p 303**

Inflammation is a well-recognized driver of atherosclerosis and in particular, macrophages, are thought to playing a central role.\(^2\) A noninvasive imaging technique that is able to detect and quantify intraplaque macrophages at different stages of plaque development and in response to therapy could be of great clinical value.

Several gadolinium-based and iron oxide–based contrast agents have been developed and investigated for the detection and characterization of inflammatory processes in atherosclerotic plaques.\(^3,4\) Iron oxide–based contrast agents appear to offer the greatest potential for short-term clinical translation because they have already been used in patients.\(^5,6\)

The ferromagnetic properties of iron oxide particles, which are internalized by and accumulate in macrophages, lead to a change in the resonance frequency of surrounding water molecules and a shortening of their relaxation times. This can be visualized as signal loss in T2- and T2*-weighted MRI images.\(^4\) The resulting hypointense signal can be detected at...
nanomolar local concentrations, yielding a significantly higher sensitivity compared with the detection threshold of gadolinium-based contrast agents.\(^7\)

Iron oxide particle detection can be ambiguous because signal loss can be mimicked by artifacts, resulting from cardiac motion, interfaces between the trachea/lung and surrounding tissue, local hemorrhage, or calcification.\(^8\) Additionally, if tissues adjacent to iron oxide accumulations do not yield high signal-to-noise ratios, like the thoracic vessels, it can be difficult to localize, delineate, and quantify the exact area of signal void.\(^9\) Several techniques such as GRASP (gradient echo acquisition for superparamagnetic particles) and IRON (inversion recovery with on-resonant water suppression) have been developed to generate positive contrast or hyperintense signal to improve detection, delineation, and quantification of iron oxide particles.\(^9,10\) Most of these methods require dedicated imaging sequences and precise knowledge about the anticipated frequency shifts and are prone to large-scale field inhomogeneities and local alternations caused by chemical shift.\(^11\)–\(^13\)

An alternative method, susceptibility gradient mapping (SGM), can be applied by postprocessing. This technique has been demonstrated to allow positive contrast visualization of iron oxide on MR gradient echo data sets.\(^14\) SGM utilizes the local shift in k-space caused by local field distortions (susceptibility gradients), resulting from the presence of iron oxide particles.\(^15\) In particular, the shift in k-space depends on the local concentration of iron oxide particles, that is, higher local concentrations result in larger shifts. SGM-MRI allows from the calculation of the shift in k-space, \(k_s\), to infer the strength of the susceptibility gradient and thus to derive information about the local concentration of iron oxide particles.

In this study, we sought to test whether iron oxide particles in macrophage-rich plaques can be visualized and quantified in vivo using a \(T_1/T_2\) ratio higher than reported for other superparamagnetic iron oxide particles or ultrasound particles of iron oxide (eg, SHU555C).\(^17\) The values for \(R^1\) and \(R^2\) (at 0.94 T) are 20.1 mmol/L×s\(^{-1}\) and 37.1 mmol/L×s\(^{-1}\), respectively. VSOP-C184 has a core diameter of 4 nm and a hydrodynamic diameter of 7.0±0.15 nm.\(^17\) In contrast to ultrasound particles of iron oxide particles, which are stabilized sterically, typically by dextran coating, VSOP particles are stabilized electrostatically by complex binding mechanisms between the citrate and the surface of the iron oxide particle.\(^18\) Phase I and II clinical data of VSOP-C184 demonstrate its potential for clinical translation.\(^6\)

**Methods**

**Animals**

Homozygous male apoE\(^{-/-}\) mice (C57BL/6J strain background) were acquired from Charles Rivers Laboratories (Edinburgh, UK) and bred within the Animal Unit of the Rayne Institute. The housing and care of the animals and all procedures used in this study were performed in accordance with the guidelines and regulations of the United Kingdom Home Office.

At 8 weeks of age, mice were switched from normal rodent chow to a high-fat diet (HFD) for 4 to 12 weeks. The HFD contained 21% fat from lard and was supplemented with 0.15% (wt/wt) cholesterol (Special Diets Services, Witham, United Kingdom). Twenty-week-old male C57BL/6J mice were used as controls. In the treatment group, pravastatin administration (40 mg/kg of body weight per day) was started at the same time as commencement of HFD feeding.

**Very Small Iron Oxide Particle Contrast Agent**

Very small iron oxide particle (VSOP)-C184 is a citrate-coated, very small superparamagnetic iron oxide particle with a \(T_1/T_2\) ratio higher than reported for other superparamagnetic iron oxide particles or ultrasound particles of iron oxide (eg, SHU555C).\(^17\) The values for \(R^1\) and \(R^2\) (at 0.94 T) are 20.1 mmol/L×s\(^{-1}\) and 37.1 mmol/L×s\(^{-1}\), respectively. VSOP-C184 has a core diameter of 4 nm and a hydrodynamic diameter of 7.0±0.15 nm.\(^17\) In contrast to ultrasound particles of iron oxide particles, which are stabilized sterically, typically by dextran coating, VSOP particles are stabilized electrostatically by complex binding mechanisms between the citrate and the surface of the iron oxide particle.\(^15,17\) Phase I and II clinical data of VSOP-C184 demonstrate its potential for clinical translation.

**Imaging Protocol**

MRI was performed in apoE\(^{-/-}\) mice at 4, 8, and 12 weeks after commencement of the HFD and in 20-week-old control animals (C57BL/6J mice). Eight mice were scanned at each time point, before contrast, and 48 hours after the initial injection of the iron oxide agent and subsequently euthanized for histological analysis. Iron oxide particles (VSOP, Charite, Berlin, Germany) were administered at a dose of 300 μmol Fe/kg via tail-vein injection 24 and 48 hours before the postcontrast MR examination (Figure 1). To demonstrate treatment effects, additional 8 apoE\(^{-/-}\) mice were scanned after 12 weeks of statin treatment.

All MRI was performed on a 3-T Achieva clinical scanner (Philips Healthcare, Best, The Netherlands) equipped with a 23-mm single-loop microscopy coil. For localization of the brachiocephalic arterty, a low-resolution gradient echo scout scan was performed in coronal and transverse orientation using the following parameters: field of view=200 mm; matrix=320; slice thickness=2 mm; TR/TE=20/5.8 ms; flip angle=30°; and slices=9. The scout scan was followed by a time-of-flight scan in the transverse orientation for visualization of the aortic arch and the brachiocephalic artery. Imaging parameters included field of view=20×20×10 mm; matrix=160×160; in-plane spatial resolution=0.3×0.3 mm (reconstructed, 0.13×0.13 mm); slice thickness=0.5 mm; TR/TE=37/7.7 ms; and flip angle=60°. From the time-of-flight data set, a maximum intensity projection was generated to display an angiogram of the aortic arch with associated vessels and to plan the subsequent vessel wall scans. An ECG-triggered, \(T_2^*\)-weighted 3D gradient echo data set was acquired with field of view=16×16×8 mm; matrix=176×176; in-plane spatial resolution=0.12×0.12 mm; slice thickness=0.5 mm; TR/TE=21.69 ms; and flip angle=25° (Figure 2).

**Susceptibility Gradient Mapping**

Recently, a technique for the estimation of field distortions has been proposed through truncation of successive lines of k-space, allowing the calculation of \(k_s\) (k-space shift) for every image pixel without compromising image resolution.\(^18\) For this, a filter line is applied along the \(k_x\) and \(k_y\) directions that subsequently nulls specific lines in k-space. The shift in k-space, \(k_s\), with respect to \(k_0\) is determined by localizing the signal minimum of corresponding image data sets. Figure 3 shows the main principle of this technique for 2 areas with...
different local concentrations of iron oxide particles resulting in susceptibility gradients with different strengths and thus different k-space shifts. The shift in k-space, \( k_s \), can be determined for each pixel by applying the filter and identifying the filter position that results in a signal minimum (Figure 3B). The value of the k-space shift can be displayed as a parameter map of susceptibility gradients for all pixels resulting in a positive contrast image (Figure 3C). Furthermore, parameter maps provide information about the local concentration of iron oxide particles, because a higher local concentration results in a larger susceptibility and thus a larger shift in k-space. As described, the local susceptibility gradient \( G_s \) can be determined from the k-space shift \( k_s \) by

\[
G_s = \frac{k_s \cdot G_i}{TE}, \quad G_i \gg G_s
\]

where \( G_i \) is the strength of the imaging gradient, TE is the echo time, and \( t \) is the sampling interval.

SGM was performed for quantification of the echo shift \( k_s \) caused by the presence of iron oxide particles and was applied to the complex data of 3D gradient echo data sets from all animals scanned. All postprocessing methods were implemented in Matlab (R2007b), and a Dicom output file including the information from the gradient echo data set was generated for analysis with Osirix image processing software (Open Source, University of Geneva).

**Supplementary Methods**

Supplementary methods on animal handling, MRI analysis, mass spectrometry, Western immunoblot analysis, and in vitro experiments are available in the online-only Data Supplement Methods online.

**Statistical Analysis**

Values are expressed as mean±SD. Differences across groups were compared by ANOVA followed by the Bonferroni post hoc correction (Systat Software, San Jose, CA). Receiver operating characteristics were used to select a cutoff value for SG parameter maps after the injection of iron oxide particles to provide an optimal trade-off between sensitivity and specificity to distinguish between control animals and apoE\(^{-/-}\) mice on an HFD. The relation between continuous variables was examined by least-squares linear regression analysis.

### Results

MRI before and after iron oxide particle administration was successfully performed in all apoE\(^{-/-}\) mice on HFD and control animals.

**T2*-Weighted MRI**

Cross-sectional T2*-weighted images of the brachiocephalic artery in control and apoE\(^{-/-}\) mice demonstrated a bright circular lumen before injection of the iron oxide agent (Figure 2 and Figure 4). After iron oxide injection, increasing regions of signal loss were observed in brachiocephalic artery plaques in apoE\(^{-/-}\) mice on 4, 8, and 12 weeks of HFD, whereas no such effect was observed in control mice. The area of signal loss was contiguous and represented a significant percentage of the luminal area. An increase in signal loss (relative to precontrast MRI scans) was observed with plaque progression after 4, 8, and 12 weeks of HFD (control: 1.7±0.8%; 4 weeks of HFD: 6.3±4.3%; \( P = 0.68 \) versus control; 8 weeks of HFD: 16.3±6.4%; \( P < 0.17 \) versus 4 weeks of HFD; 12 weeks of HFD: 30.5±6.8%; \( P < 0.001 \) versus 8 weeks of HFD). In statin-treated animals, a less pronounced signal loss was observed after 12 weeks of HFD (8.4±7.5%, \( P < 0.001 \) versus 12 weeks of HFD). Animals were euthanized after the final MRI scan.

**Susceptibility Gradient Mapping**

Complex data from T2*-weighted images were postprocessed, and SG parameter maps were generated. Because of the well-defined and clearly delineated areas of positive contrast on SG parameter maps, image analysis was feasible without precontrast scans (Figures 2 and 4). Precontrast SGM values (day 0, Figure 1) were measured on scans before the injection of an iron oxide particle for both control and apoE\(^{-/-}\) mice on HFD (control: 3.71±1.26 mT/m; 4 weeks of HFD: 3.04±1.04 mT/m; 8 weeks of HFD: 3.42±1.04 mT/m; 12 weeks of HFD: 3.76±1.74 mT/m; statin: 3.89±1.08 mT/m; \( P > 0.05 \) between groups). SGM values measured in the control group 48 hours after the initial injection did not differ significantly from values of precontrast SG parameter maps of any group (\( P > 0.05 \)). Forty-eight hours after the initial injection (Figure 1) of iron oxide particles, SGM values increased with plaque progression (Figure 5A). In statin-treated animals, SGM values significantly differed (\( P < 0.001 \)) from the 12-week group (Figure 5A). Animals were euthanized after the final MRI scan. On a

**Figure 2.** A1 and B1. In vivo time-of-flight imaging of the aortic arch in control and apoE\(^{-/-}\) mice (aA indicates ascending aorta; dA, descending aorta; BC, brachiocephalic artery; SC, subclavian artery; and CA, carotid artery). The subsequently performed imaging sequences were aligned perpendicular to the brachiocephalic artery. The aortic arch and BC artery were first scanned using a low-resolution, bright-blood sequence. A2–3 and B2–3. Based on these scans, bright-blood, high-resolution scans were planned (precontrast: A2, B2; after iron oxide: A3, B3) including the complex image data. A4–5 and B4–5. From these scans, SGM-positive contrast images were derived (precontrast: A4, B4; after iron oxide: A5, B5, A6, B6).
plaque iron oxide deposition (Figure 5). The absolute iron content in the brachiocephalic artery was relatively low in control mice (460±99 mg/L). After the initiation of HFD, a significant increase in iron oxide levels was observed (4 weeks of HFD: 795±164 mg/L; 8 weeks of HFD: 2138±608 mg/L; 12 weeks of HFD: 4060±763 mg/L). When a statin was administered simultaneously with the HFD, a considerably lower iron oxide content (1377±381 mg/L) was measured. Without prior VSOP injection, iron oxide content in animals on a 12-week HFD was not significantly different from the control group (427±134 mg/L versus 460±99 mg/L, P > 0.05). A significant correlation between the absolute iron oxide content in the brachiocephalic artery by ICP-MS and values from SG parameter maps (R²=0.70, P < 0.05, Figure 5B) was found.

Histology, Immunohistochemistry, Electron Microscopy, and Western Immunoblotting
The positive staining for Mac-2 (Figure 4) demonstrated the presence of macrophages in atherosclerotic lesions. We observed an increase in %Mac-2 and %Perls stain with increasing duration of the HFD (R²=0.72, P < 0.05, Figure 6). In the statin group, a slight decrease in the area positive for macrophages was observed, whereas there was a pronounced decrease in the area positive for iron. Without prior VSOP injection, Perls stain was negative for iron oxide deposition in mice with 12 weeks on HFD. Electron microscopy and high-resolution light microscopy of plaques confirmed intracellular accumulation of iron oxide particles (Figure 7) in animals on 12-week HFD.

To quantify the amount of Mac-2 expression (as a surrogate marker for macrophages) in the brachiocephalic artery plaque, we performed Western immunoblotting (Figure 8) and found a gradual and significant increase of Mac-2 antigenicity during the 12-week time course of HFD (Figure 8). There was a significantly lower Mac-2 content in plaques of 12-week HFD animals treated with a statin compared with animals on 12 weeks of HFD alone (P < 0.002, Figure 8).

In Vitro Experiments
Increasing numbers of macrophages (0.1, 0.2, 0.5, 1, and 1.5 million cells; IC-21 cell line) were incubated with 50 μL/mL of VSOP in gelatin (5%). In vitro, a good correlation (R²=0.96, P < 0.05, Figure 7C) between increasing concentrations of iron oxide (determined by ICP-MS, mg/L) and the magnitude (mT/m) of the contrast enhancement on SG parameter maps could be observed. A good correlation (R²=0.96, P < 0.05, Figure 7D) between R²* (s⁻¹) and SG parameter maps (mT/m) was found as well. Furthermore, a good correlation (R²=0.98, P < 0.05, Figure 7E) was also observed between the number of macrophages (IC-21 cell line) and the magnitude (mT/m) of the contrast enhancement on SG parameter maps.

Discussion
In this study, we successfully demonstrate the visualization and quantification of increasing plaque iron oxide uptake with the progression of plaque development in an apoE⁻⁻⁻/⁻⁻⁻
mouse model using SGM-MRI. The magnitude (mT/m) of contrast enhancement on SG parameter maps was in good agreement with results from ICP-MS, histological staining for iron, and macrophages. This study demonstrated that SG parameter maps provide useful complementary information to T2*-weighted images for the characterization of intra-plaque iron oxide depositions.

Although other groups have already studied iron oxide uptake in atherosclerosis in preclinical and clinical settings, several findings of this study are novel.

1. Iron oxide accumulation can be assessed noninvasively in the brachiocephalic artery of apoE<sup>−/−</sup> mice at clinically relevant field strengths (3 T) using clinically applicable MRI sequences.

2. Atherosclerotic lesions in the brachiocephalic artery of apoE<sup>−/−</sup> mice can be accurately detected and visualized in vivo by positive contrast at different stages of plaque development after systemic administration of the iron oxide particles using SG parameter maps.

3. The application of SG parameter maps allows the quantification of iron oxide particles in atherosclerotic plaques using the magnitude (mT/m) of the contrast enhancement. Furthermore, the effect of anti-inflammatory therapy could be detected and quantified using SG parameter maps, without increasing scan time or the necessity for comparison with precontrast MRI scans (as required for the analysis of T2*-weighted images).

Several superparamagnetic particles are currently in or have completed phase I-III clinical trials in the United States and Europe and therefore should potentially be available for broader clinical use in the near future.

A model in which the development of atherosclerotic plaques is reproducible and resembles human disease has great utility when evaluating the efficacy of novel contrast agents, aimed at the detection and quantification of molecular changes during the progression of atherosclerosis. The most commonly used model is the apoE<sup>−/−</sup> mouse. Recent reports implied that the brachiocephalic artery of the apoE<sup>−/−</sup> mouse model exhibits a consistent rate of lesion development/progression and that lesions in this vessel segment exhibit many properties that are relevant to the development of clinically significant disease. This model is therefore suitable for the investigation of iron oxide uptake at different stages of plaque development.
Despite the prevalence of high-field MR scanners in previously published studies in rodents, clinical MRI systems are being increasingly used for preclinical research. This study was performed on a clinical 3-T MR scanner for several reasons. The effects of iron oxide particles and potential sources of artifacts (fat, air/tissue interfaces) on T2*-weighted images and SGM-MRI can significantly vary between field strength (1.5 T to 3 T versus >7 T). Additionally, when using a clinical MRI system, protocols and imaging pulse sequences that are already optimized for clinical practice can be adapted, developed further, and scaled down for preclinical use. Subsequently, these sequences can

![Diagram of SGM-MRI signal enhancement and correlation with quantitative Fe analysis of brachiocephalic artery samples]

Figure 5. Time course of SGM-MRI signal enhancement (A) and correlation with quantitative Fe analysis of brachiocephalic artery samples (B). Quantitative analysis of Fe content in the brachiocephalic artery was performed by ICP-MS analysis after the final MR scan. Histology (C) and immunohistochemistry data (D) of the brachiocephalic artery. Data (C) are presented as %Perls stain per vessel area. Correlation between %Perls stain per vessel area and Mac-2 to vessel area is shown in D.

Figure 6. A, Good correlation between SGM signal and Mac-2 density was observed in brachiocephalic artery plaques (control and 4, 8, and 12 weeks) in matched slices. B, Cutoff value for the SGM signal of 4.44 mT/m resulted in a sensitivity of 88% and a specificity of 92% to distinguish between control animals and apoE−/− mice on a HFD.
be scaled back for clinical use. The translation from preclinical to clinical studies is therefore facilitated; 3 T was chosen over 1.5 T because of the increased signal to noise and thus higher spatial resolution achievable.

MRI in Atherosclerosis Using T2- and T2*-Weighted MRI
Ruehm et al were the first to report that iron oxide particles are taken up by macrophages in atherosclerotic plaques and can be imaged by MRI. Since then, a number of studies have been carried out using iron oxide particles with the aim of detecting macrophages in atherosclerotic plaque in preclinical and clinical settings. Iron oxide particles could be detected as a result of the shortening of the local T2 and T2* relaxation times, creating a detectable signal void.

The main limitations of negative contrast approaches are, (1) In anatomic regions with low intrinsic signal-to-noise ratios or in the absence of surrounding tissue, it can be difficult to detect and delineate areas of iron oxide accumulation. (2) The presence of motion artifacts, hemorrhage, and calcification can cause negative contrast effects not distinguishable from signal voids caused by iron oxide particles. In this study, image interpretation of T2*-weighted images after the injection of VSOP has been challenging, because of the mentioned limitations (signal voids from, eg, the trachea adjacent to the brachiocephalic artery). It was only possible to accurately detect and delineate iron oxide uptake (% signal void) if precontrast T2*-weighted MRI scans were taken as a reference.

SG Parameter Maps
Several positive-contrast techniques have been developed and investigated in recent years and have demonstrated unique potential to improve the sensitivity for in vivo detection of...
iron oxide particles. Three positive contrast methods (SGM, IRON, and GRASP) received particular attention in recent publications. All techniques allowed detection of iron oxide-labeled cells as hyperintense voxels. Furthermore, the positive contrast generated by these techniques was shown to provide greater sensitivity for the detection of iron oxide depositions compared to the negative contrast provided by T2*-weighted images.

Because positive-contrast images specifically highlight areas of susceptibility gradients or resonance frequency shifts, they do not yield detailed anatomic information. Additional scans are therefore required to obtain data sets yielding anatomic information. Both white Marker (GRASP) and IRON techniques require detailed knowledge of expected susceptibility gradients or frequency shifts and dedicated pulse sequence design. Using SGM-MRI, no additional data acquisition or dedicated pulse sequence design is needed. SGM-MRI is a postprocessing technique that can be applied to any 2D or 3D gradient-echo data set. Another advantage of SGM-MRI is the selective suppression of susceptibility artifacts (eg, air-tissue interfaces) existing in these regions.

In contrast to T2*-weighted MRI, in which areas of the signal void depend on local iron oxide concentrations and applied imaging parameters (especially the echo time, TE), SGM-MRI allows the calculation of a local susceptibility gradient. This gradient value is independent from parameters such as the TE because it is taken into consideration in the calculation of susceptibility gradient-maps. Therefore, SGM-MRI provides a “physical” property, which is proportional to the local amount of iron oxide and independent from imaging parameters. Only the accuracy of the SGM estimation depends on the parameter values. Even though it is possible to estimate local iron oxide concentrations by relaxometry, for example, T2* mapping, such techniques are time-consuming and thus difficult to apply in vivo. In contrast, SGM-MRI estimates local susceptibility gradients by using the imaging data itself without modifying and prolonging the image acquisition. SGM-MRI may therefore represent a fast, clinically applicable method, which could allow the quantification of the iron oxide accumulation in atherosclerotic plaques.

**Study Limitations**

The total dose of the iron oxide injected into animals was 600 μmol Fe/kg, comparable to previous preclinical experiments, but higher than in clinical studies. Future studies will be directed toward dose optimization because this was a proof-of-concept study, investigating the sensitivity and specificity of SGM-MRI for the detection and quantification of iron oxide particles in atherosclerotic plaques. Positive contrast on SG parameter maps resulted from superparamagnetic iron oxide particles and may be the result of recruitment of VSOP-labeled blood-born monocytes into inflamed “shoulders” of atherosclerotic plaques. Alternatively, VSOP may have entered plaques as the result of increased endothelial permeability or through leaky neovessels followed by subsequent phagocytosis of plaque resident macrophages. The exact mechanism of iron oxide uptake into atherosclerotic plaques is yet not fully elucidated and remains subject to further investigations. Other sources of high parameter values on the SG parameter maps may result from, for example, endogenous iron oxide in areas of intraplaque hemorrhage. In this study, no positive contrast was observed before VSOP injection on SG parameter maps. Because of the study design, the blood half-life of VSOP particles for the injected dose and the effect of VSOP particles on the inflammatory environment in the atherosclerotic vessel wall were not investigated in this study. The safety of iron oxide nanoparticles, including VSOP, has been evaluated in humans, confirming that these agents have an acceptable safety profile for use in humans.

**Conclusion**

SGM-MRI allowed the sensitive detection and quantification of intraplaque iron oxide particles in a mouse model of progressive atherosclerosis after VSOP injection. Iron oxide particles colocalized with plaque resident macrophages and could be displayed with high spatial resolution. The magnitude (mT/m) of SGM enhancement demonstrated a good correlation with the amount of intraplaque iron oxide particles confirmed by ICP-MS and histology. SGM-MRI could be useful for the in vivo detection and quantification of iron oxide particle uptake into macrophage-rich plaques.

**Sources of Funding**

This study was funded by the British Heart Foundation project grant (PG/09/061) awarded to Dr Botnar.

**Disclosures**

The MRI scanner is partly supported by Philips Healthcare. Dr Wiethoff is an employee of Philips Healthcare. The contrast agent was provided by the Charite, Berlin, Germany.

**References**

CLINICAL PERSPECTIVE

Coronary artery disease remains the leading cause of death in the Western world. The majority of complications result from plaque rupture and subsequent thrombosis. X-ray angiography allows the assessment of the extent of luminal narrowing but provides little information on plaque composition and biology, resulting in the inability to distinguish between stable and unstable lesions. Inflammation is a recognized contributor to atherosclerotic plaque development and complication. Macrophages are thought to play a central role in these processes. Therefore, a noninvasive imaging technique that is able to detect and quantify intraplaque macrophages could be of great clinical value. Using “standard” T2- or T2*-weighted sequences, iron oxide detection can be ambiguous because signal loss can be mimicked by artifacts resulting from cardiac motion, interfaces between tissues, local hemorrhage, or calcification. Susceptibility gradient mapping allows positive contrast visualization of iron oxide-labeled stem cells using inversion-recovery with on-resonant water suppression (iron). 

MRI could be useful for the in vivo detection and quantification of iron oxide uptake into macrophage-rich plaques and to monitor response to treatment in patients with atherosclerotic vessel wall disease.
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*Circ Cardiovasc Imaging*. 2011;4:295-303; originally published online March 21, 2011; doi: 10.1161/CIRCIMAGING.110.957209

*Circulation: Cardiovascular Imaging* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

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Print ISSN: 1941-9651. Online ISSN: 1942-0080

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Supplementary material

Supplemental Methods

Anesthesia and Euthanasia

Animals were anesthetized by an intramuscular injection of a combination of Medetomidin (500 µg/kg), Midazolam (5 mg/kg), and Fentanyl (50 µg/kg). A reversal agent Atipamezol (2.5 mg/kg), Flumazenil (500 µg/kg), and Naloxone (1200 µg/kg) was administered for serial imaging experiments.¹

Terminal exsanguination was performed by arterial perfusion via the abdominal aorta with PBS at a constant pressure of 100 mm Hg, with outflow through the right atrium. This was followed by constant pressure perfusion in situ with 10% formalin when vessels were to be embedded for histology. Brachiocephalic arteries were removed with a piece of the aortic arch and the stump of the right subclavian artery still attached to aid orientation during histological processing. Pieces of the aortic arch, of the right subclavian artery as well as the carotid artery were removed for Inductively Coupled Plasma Mass Spectroscopy (ICP-MS).

Animal handling and monitoring

After induction of anesthesia the animal was positioned in prone position on a 23-mm surface coil (Philips Healthcare, Best, NL). Temperature was monitored continuously and maintained at 37°C using a small rodent heater system (SA Instruments, Stony Brook, NY). An electrocardiogram (ECG) signal was derived from two electrodes placed subcutaneously on the left and right side of the mouse chest. Synchronization of the MR
imaging sequences with the ECG was achieved using a small animal gating system (SA Instruments, Stony Brook, NY) and allowed to obtain end diastolic vessel wall images free of motion artifacts.

**Statin treatment**

Pravastatin (sodium salt, Kemprotec Limited, Middlesbrough, UK) was administered to a group of ApoE⁻/⁻ mice in their drinking water (ordinary tap water) at a dose of 40mg/kg of body weight per day. Animals started to receive Pravastatin at the same time as commencement of HFD feeding. MRI was performed 12 weeks later (Figure 1). Age-matched HFD fed animals not given a statin were used as controls.

**MR image analysis**

Analysis was performed using Osirix image processing software (Open-Source, University of Geneva). In vivo image analysis was performed on cross-sectional T2*-weighted as well as SG (Susceptibility Gradient) parameter-maps of the brachiocephalic and carotid artery.

**Quantification of T2*-weighted MRI**

All data derived from T2*-weighted images are presented as the signal loss area (%) with respect to the pre-contrast MRI scan. Lumen areas of the brachiocephalic artery were compared between pre-contrast T2*-weighted MR images prior to iron oxide injection and the MR images 48h post injection of iron oxide. Identical settings for window and
level were used to compare the areas of signal void within the ROI. Areas were determined semi-automatically using identical 2D segmentation parameters.

**Quantification of SG parameter-maps**

To quantify SG parameter-maps of the brachiocephalic wall, measurements of the mean susceptibility gradient were performed in ROIs encompassing the area of high parameter values within the vessel wall without the need for comparison with the native MRI scan.

**Mass spectroscopy**

ICP-MS was performed on a subset of plaque samples. Vessel samples were digested in 70% nitric acid at 37°C overnight followed by dilution with deionized water for ICP-MS analysis. A standard curve was run with each sample set for Fe concentration determination.

**Histology and Immunohistochemistry**

Brachiocephalic arteries were embedded in paraffin. Paraffin sections (5µm) were stained using Haematoxylin and Eosin (H&E), Miller’s Elastin/van Giesson (EvG) stain and Perl’s Prussian Blue Stain. Immunohistochemical location of macrophages using an antibody against galectin 3 (rat anti mouse Mac-2, Biolegend) required an initial epitope retrieval using 10mM citrate pH6.5, in pressure cooked for 10mins. Retrieved sections were blocked for 10mins at room temperature with DAKO block. Sections were then incubated at room temperature for 45mins in primary antibody (1:500), followed by 3 washes with DPBS, pH 7.4. Mac-
2 binding was located by incubation with polyclonal rabbit anti-rat IgG:Biotin (DAKO, 1:100) followed by an extravidin-peroxidase complex (Vector Laboratories), and peroxidase substrate, SG (Vector Laboratories). Slides were washed, counterstained with Nuclear Fast Red (Vector Laboratories) and mounted.

**Plaque Morphometry**

To co-register MR images with histological sections the brachiocephalic artery was removed with the adjacent part of the aortic arch. Sections were cut along the brachiocephalic artery (starting from the proximal end). Landmarks for co-registration were the aortic arch and the subclavian artery. A time of flight (TOF) angiogram was acquired before planning of the imaging slices. Based on the 3D maximum intensity projection (MIP) reconstructed from the TOF angiograms, imaging slices could be planned exactly perpendicular to the course of the brachiocephalic and carotid artery, starting at the branching point with the aortic arch. The analyzed imaging slice was planned to include only the proximal part of the brachiocephalic artery, distal to the bifurcation with the aortic arch.

Analyses were made on digitised images of EvG, Perl’s and Mac2-stained sections. Morphometry as well as quantification of area of staining was measured using computer-assisted image-analysis (Image Pro Plus, Media Cybernetics, Inc., MD). Co-registration of Mac-2 and Perl’s positive staining areas was assessed in serial sections of atherosclerotic lesions.

**Immunoblot analysis**
Brachiocephalic arteries (two imaged and one additional control as well as ApoE \(-/-\) mice per group) were excised and frozen in liquid nitrogen. Samples were homogenized in protein extraction buffer (50 mmol/L Tris-HCl; pH 7.5, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% (w/v) Triton X-100, 0.1 % \(\beta\)-mercaptoethanol, 1 mmol/L sodium orthovanadate, 50 mmol/L sodium fluoride, 5 mmol/L sodium pyrophosphate and protease inhibitor tablets (Complete®, Rosh Applied Science). One ml of extraction buffer was used per 100mg of vascular tissue. The homogenates were centrifuged at 4°C for 12min at 15,000g to remove and the supernatant used for electrophoresis by SDS-PAGE. Nitrocellulose membranes were blocked with 5% non-fat milk, and incubated with primary antibodies (rat anti-Mac-2-antibody, 1/500 diluted, BioLegend). The membranes were developed using biotinylated rabbit anti-rat IgG (DAKO, diluted 1/2000). ExtrAvidin diluted 1/2000 and proteins visualized by chemiluminescence (ECL, Amersham). Bands were quantified using western blot analysis, ImageJ software based analysis (http://rsb.info.nih.gov/ij/), with the area under the curve (AUC) of the specific signal calculated.

**In-vitro experiments**

The IC-21 mouse macrophage cell-line was used for these experiments. Increasing numbers of IC-21 macrophages (0.1, 0.2, 0.5, 1, 1.5 million cells) were incubated for 2hr with 50 \(\mu\)l/ml of iron oxide.\(^2\) The cells were washed with PBS, incubated for another hour with 50 \(\mu\)l/ml of iron oxide and washed again in order to remove excess iron oxide. A cell count was obtained post-incubation using a haemocytometer. Iron oxide concentration was determined by ICP-MS after the experiment. MRI with the following
parameters comparable to the in vivo experiment was performed: flip angle=25º, FOV = 16x16x8mm, matrix = 176x176x20, in-plane resolution = 120 µm x 120 µm; slice thickness = 0.5 mm, TE=6.9ms and TR=21ms. As control the same set of concentrations of macrophages were imaged without the addition of iron oxide at incubation. In additional T2* map relaxometry was performed using 6 echoes (first echo: 3.4ms, echo spacing 5.7ms) allowing comparison to an additional quantification method.³
References

