Molecular Imaging

Increased Vascular Permeability Measured With an Albumin-Binding Magnetic Resonance Contrast Agent Is a Surrogate Marker of Rupture-Prone Atherosclerotic Plaque

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Background—Compromised structural integrity of the endothelium and higher microvessel density increase vascular permeability. We investigated whether vascular permeability measured in vivo by magnetic resonance imaging using the albumin-binding contrast agent, gadofosveset, is a surrogate marker of rupture-prone atherosclerotic plaque in a rabbit model.

Methods and Results—New Zealand white rabbits (n=10) were rendered atherosclerotic by cholesterol-diet and endothelial denudation. Plaque rupture was triggered with Russell’s viper venom and histamine. Animals were imaged pre-triggering, at 3 and 12 weeks, to quantify plaque area, vascular permeability, vasodilation, and stiffness and post-triggering to identify thrombus. Plaques identified on the pretrigger scans were classified as stable or rupture-prone based on the absence or presence of thrombus on the corresponding post-trigger magnetic resonance imaging, respectively. All rabbits had developed atherosclerosis, and 60% had ruptured plaques. Rupture-prone plaques had higher vessel wall relaxation rate (R1; 2.30±0.5 versus 1.86±0.3 s⁻¹; P<0.001), measured 30 minutes after gadofosveset administration, and higher R1/plaque area ratio (0.70±0.06 versus 0.47±0.02, P = 0.01) compared with stable plaque at 12 weeks. Rupture-prone plaques had higher percent change in R1, between the 3 and 12 weeks compared with stable plaque (50.80±7.2% versus 14.22±2.2%; P<0.001). Immunohistochemistry revealed increased vessel wall albumin and microvessel density in diseased aortas and especially in ruptured plaque. Electron microscopy showed lack of structural integrity in both luminal and microvascular endothelium in diseased vessels. Functionally, the intrinsic vasodilation of the vessel wall decreased at 12 weeks compared with 3 weeks (18.60±1.0% versus 23.43±0.8%; P<0.001) and in rupture-prone compared with stable lesions (16.40±2.0% versus 21.63±1.2%; P<0.001). Arterial stiffness increased at 12 weeks compared with 3 weeks (5.00±0.1 versus 2.53±0.2 m/s; P<0.001) both in animals with stable and rupture-prone lesions.

Conclusions—T1 mapping using an albumin-binding contrast agent (gadofosveset) could quantify the changes in vascular permeability associated with atherosclerosis progression and rupture-prone plaques. (Circ Cardiovasc Imaging. 2016;9:e004910. DOI: 10.1161/CIRCIMAGING.116.004910.)

Key Words: atherosclerosis □ capillary permeability □ cardiovascular diseases □ magnetic resonance imaging □ models, animal

Despite the successful implementation of several primary and secondary prevention strategies, atherosclerosis remains a crucial causal factor underlying stroke and myocardial infarction, which are the major causes of mortality and morbidity in Europe and the United States.1,2 Rupture of unstable atherosclerotic lesions is a major factor in the development of thromboembolic sequelae after atherosclerosis and symptomatic disease.3,4 Damaged vascular endothelium and increased microvessel density lead to leakage of plasma components, including albumin, fibrinogen, erythrocytes, lipids, and inflammatory cells into the vessel wall, and accelerate disease progression and lesion instability.4,5

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Vascular endothelium controls both vascular permeability, by regulating the flux of blood components through the vessel wall via intracellular and intercellular pathways,6 and vascular tone by balancing the production of vasodilators (eg, nitric oxide and prostacyclin) and vasoconstrictors (eg, endothelin-1

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and angiotensin II). However, cardiovascular risk factors impair both the structural integrity and functionality of endothelial cells (ECs), which render the vessel wall proatherogenic by increasing endothelial permeability and attenuating endothelium-dependent vasodilation. 5, 9, 10 Additionally, plaque growth, local hypoxic conditions, and inflammation stimulate microvessel growth within the plaque. 5, 11 MicrovesSEL density correlates with the extent of the necrotic core, intraplaque hemorrhage, 5, 12 and inflammatory infiltrates 13, 14 and is higher in ruptured than in stable lesions in both the coronary 4, 15 and carotid arteries. 5, 13–18 Structurally, intraplaque and adventitial microvessels are immature, dysmorphic, and fragile. They lack surrounding α-actin–positive mural cells and are covered by structurally compromised ECs, which make them more leaky and permeable. 11, 13, 14, 16, 18, 19

Dynamic contrast enhanced–magnetic resonance imaging (MRI) using nonspecific contrast agents, eg, Gd-DTPA, has extensively been used to study plaque angiogenesis. Measurements of the contrast agent kinetic parameters such as the transfer constant (Ktrans) or the area under the signal intensity curve showed correlation of contrast uptake with microvascular density. 20–23 adventitial vasa-vasorum, 24, 25 and with sites of active inflammation 26 in both animal models and man. We 27–29 and others 21–23, 30 have used the albumin-binding MRI contrast agent, gadofosveset, to image vascular remodeling and treatment response in experimental and human atherosclerosis. Collectively, these studies show that gadofosveset uptake occurs through damaged endothelium or microvessels and may be a surrogate marker of vascular permeability. Gadofosveset is a clinically approved gadolinium-based agent. Approximately 70% of injected gadofosveset binds reversibly to plasma albumin that increases its r1 relaxivity by 5–to 10-fold (r1=25 mmol L−1 s−1) compared with the free fraction (r1=5 mmol L−1 s−1). 31 In this study, we used the MR albumin-binding contrast agent, gadofosveset, and T1 mapping in a rabbit model of atherosclerosis and controlled plaque rupture to investigate whether increased vascular permeability associates with plaque progression and rupture-prone plaque. This kind of noninvasive assessment of vascular leakage could provide both diagnostic and prognostic information for disease stratification.

**Methods**

**Animal Model**

Ten New Zealand white rabbits (3 months old, male, 2.5 kg; Harlan, United Kingdom) were fed a 1% cholesterol-diet (Special Diet Services, United Kingdom) for 2 weeks before and 6 weeks after aortic denudation. This was followed by 4 weeks of normal chow diet (Figure I in the Data Supplement). 5, 19 Aortic denudation was performed under general anesthesia with acepromazine (0.75 mg/kg IM), ketamine (35 mg/kg IM), and xylazine (5 mg/kg IM), and maintained with isofluorane of 1%–2%. A 3F Fogarty catheter was introduced in the aorta through a right femoral artery cutdown. The catheter was first advanced to the level of the diaphragm, inflated with saline, and was retracted to the femoral artery. This procedure was repeated 3×. Subsequently, the catheter was removed, and the incision was closed.

At the end of the 12 weeks, diseased rabbits were injected with Russell viper venom (0.15 mg/kg IP; Enzyme Research Laboratories, Swansea, United Kingdom), a procoagulant factor, followed 30 minutes later by histamine, a vasoconstrictor in rabbits (0.02 mg/kg IV; Sigma-Aldrich, MO), to induce plaque rupture and thrombosis. This procedure was repeated twice within 4 hours. Four, nonballon-injured, age- and sex-matched rabbits (3 months old, male) were fed a normal diet for 12 weeks and used as controls. All procedures were approved by the United Kingdom Animal (Scientific Procedures) Act 1986.

**In Vivo MRI**

The abdominal aorta of New Zealand white rabbits was imaged using a 3T Philips Achieva MR scanner (Philips Healthcare, Best, The Netherlands) and a 32-channel cardiac coil. Animals were imaged at 3 time points: 3 and 12 weeks after initiation of the experimental protocol (pretrigger scans) and 24 hours after the pharmacological triggering (post-trigger scan; Figure I in the Data Supplement).

The pretrigger MRI included acquisition of native scout, phase-contrast MR angiography, and T1-weighted black-blood (T1wBB) images. The phase-contrast MR angiography images were acquired for visualization of the aorta, the renal branches, and the iliac bifurcation with repetition time (TR)=20 ms, echo time (TE)=3 ms, flip angle=15°, field of view (FOV)=300×150 mm, acquired matrix=256×122, resolution=1.2×0.61 mm, slices=20, Venc=150 cm/s, and averages=2. The maximum intensity projection images were used to plan the subsequent scans. ECG-triggered 2-dimensional (2D) T1wBB images were acquired with TR/TE=2 heartbeats/5.4 ms, profile order=centric, echo train length=6, BB delay=350 ms, FOV=120×85 mm, acquired matrix=384×270, resolution=0.31×0.31 mm, slice thickness=4 mm, slices=25, and averages=2. Subsequently, gadofoveset (0.03 mmol/kg; Ablavar, Lantheus Medical Imaging, North Billerica) was administered intravenously. Immediately and up to 30 minutes after contrast injection, steady state free precession cine and quantitative phase contrast angiography (QFlow) images were acquired to measure functional parameters of the vessel wall. Two-dimensional retrospectively ECG-triggered steady state free precession images were acquired with TR/TE=7.8/3.9 ms, flip angle=60°, FOV=85×121 mm, acquired matrix=172×241, resolution=0.5×0.5 mm, slice thickness=5 mm, slices=11, heart phases=18, and averages=3. Three-dimensional ECG-triggered fast gradient echo QFlow images were acquired with TR/TE=5.2/2.7 ms, flip angle=10°, FOV=80×58 mm, acquired matrix=300×141, resolution=0.4×0.4 mm, slice thickness=4 mm, slices=10, and averages=1. Two stacks of T1 mapping sequences were acquired to cover a total length of 80 mm in the foot-head direction.

T1 mapping images were acquired 30 minutes after gadofoveset administration using a Look-Locker–based gradient-echo sequence that uses a nonselective inversion pulse with inversion times ranging from 20 to 2000 ms, followed by 8 segmented readouts for 8 individual images for each of the 2 Look-Locker trains. In total, 16 images per slice were acquired. T1 mapping parameters were as follows: TR/TE=5.2/2.7 ms, flip angle=10°, FOV=80×58 mm, acquired matrix=300×141, resolution=0.4×0.4 mm, slice thickness=4 mm, slices=10, and averages=1. Two stacks of T1 mapping sequences were acquired to cover a total length of 80 mm in the foot-head direction. Finally, the post-triggering MRI session was performed 24 hours after the pretrigger scan and included acquisition of scout, phase-contrast MR angiography, and native T1wBB images to visualize thrombus. Only the diseased rabbits underwent post-triggering MRI.

After the final MRI session, rabbits received heparin (1000 USP units IV; Sigma-Aldrich) to prevent postmortem blood clotting and were euthanized with a bolus injection of sodium pentobarbital (100 mg/kg IV). The aortas were marked with suture ligature at distances above and below the left renal branch to match the total length imaged in vivo. The total length was measured with a ruler and the vessel was marked on its anterior site at every 2 cm with tissue dye. After extraction, the ligatures were used to reextend the aortas to their physiological length with the ink marks used to stretch the vessel equally. Subsequently, the aorta of each animal was divided into segments that were allocated for different ex vivo studies. A total of 36 diseased vascular segments containing 21 stable and 15 ruptured plaques and 4 control segments were analyzed. Aortic segments allocated for light microscopy (n=18: 2 control, 10 stable, and 6 ruptured plaques) and inductively coupled plasma (ICP)
studies (n=14: 2 control, 6 stable, and 6 ruptured plaques) were snap frozen and stored in −80°C. Aortic segments used for electron microscopy (Transmission Electron Microscopy [TEM]; n=8 [2 control, 3 stable, and 3 ruptured plaques], x-ray [n=2]) studies were fixed in 2% gluteraldehyde in 0.1 mol/L sodium phosphate buffer as described below.

### MRI Analysis

Pretrigger images acquired at 3 and 12 weeks were used to quantify the plaque area (PA), vascular permeability, aortic vasodilation, and stiffness. The vessel wall was manually segmented by tracing the adventitial and the lumen contours on T1wBB images using the software Osirix (OsiriX Foundation, Geneva, Switzerland). PA was calculated as

\[
PA = \text{vessel area ~ lumen area}
\]

Changes in PA between the 3 and 12 weeks were calculated using the T1wBB images based on the following formula:

\[
\%\Delta T1wPA = \frac{(T1wPA_{3weeks} - T1wPA_{12weeks})}{T1wPA_{3weeks}} \times 100
\]

T1 mapping images were used to calculate the vessel wall relaxation rate (R₁) on a pixel-by-pixel basis using a 3-parameter curve fitting procedure of the longitudinal magnetization including a T1 correction with an in-house software (Matlab, Natick, MA) 30 minutes after administration of gadofosveset as previously published and further explained in the Data Supplement.

Changes in vascular permeability between the 3 and 12 weeks were calculated based on the following formula:

\[
\%\Delta R₁ = \frac{(R₁_{12weeks} - R₁_{3weeks})}{R₁_{3weeks}} \times 100
\]

Retrospectively ECG-triggered steady state free procession images were used to calculate the intrinsic vasodilation of the vessel wall. The end-diastolic and end-systolic areas were manually segmented, and the vasodilation of the aorta was calculated based on the formula:

\[
%\text{Vasodilation} = \frac{(ED - ES)}{ED} \times 100
\]

Changes in aortic vasodilation between the 3 and 12 weeks were calculated based on the following formula:

\[
%\Delta\text{Vasodilation} = \frac{(\text{Vasodilation}_{12weeks} - \text{Vasodilation}_{3weeks})}{\text{Vasodilation}_{3weeks}} \times 100
\]

Pulse-wave velocity (PWV) was used to characterize arterial stiffness. Qflow images were analyzed using ViewForum (Philips Healthcare, Best, The Netherlands), and PWV was calculated based on the formula:

\[
\text{PWV} (\text{m/s}) = \frac{(\Delta x)}{(\Delta t)},
\]

where Δt is the time to peak velocity, Δx is the distance between the proximal (immediately inferior to the left renal branch) and distal (immediately superior to the iliac bifurcation) slices of the imaging volume.

Changes in the PWV between the 3 and 12-weeks were calculated based on the following formula:

\[
%\Delta\text{PWV} = \frac{(\text{PWV}_{12weeks} - \text{PWV}_{3weeks})}{\text{PWV}_{3weeks}} \times 100
\]

Post-trigger images were used to identify the presence or absence of mural thrombus and to retrospectively classify the corresponding lesions, identified on the 12-week pretrigger scan, in stable and rupture-prone plaques, respectively. The distances from the renal branches and the iliac bifurcation were used as internal anatomic markers to match the MRI and histologic sections. Immunohistochemistry was performed using biotinylated primary anti-human sheep polyclonal antibody for albumin (1:50; Abecam ab8940, Cambridge, MA) and mouse monoclonal antibody for CD31 (1:50; DAKO JC70A, Cambridgeshire, United Kingdom) to locate the endothelium. Primary antibody binding was located using an avidin–peroxidase complex (Vector Laboratories, Burlingame, CA). Immunopositive areas were analyzed on digital images by computerized planimetry using ImageJ (National Institutes of Health). The immunopositive area (albumin) was segmented on the images and expressed as a percentage of the total PA. Plaque microvessels (immunopositive area for CD31) were counted individually, and microvessel density was calculated by measuring the total number of microvessels and dividing by total PA.

### Transmission Electron Microscopy

The structural integrity of luminal and microvascular endothelium was studied with TEM. Aortic segments (n=8: 2 control, 3 stable, and 3 ruptured plaques) were fixed in 2% gluteraldehyde in 0.1 mol/L sodium phosphate buffer (pH 7.4) for 2 hours, washed with sodium phosphate buffer for 2 hours, and postfixed in 1% OsO₄ for 2 hours. Samples were dehydrated through a graded series of ethanol and embedded in epoxy resin. Semithin sections (0.2 μm) were stained with toluidine blue for light microscopy examinations and were used to guide sampling for TEM studies. Thin sections (0.09 μm) were collected on 150-mesh copper grids and double stained with uranyl acetate and lead citrate for electron microscopy examinations (H7650; Hitachi, Tokyo, Japan).

### Electron Probe x-Ray Microanalysis

Aortic segments (n=2: stable plaque) were prepared by cryofixation under liquid nitrogen. Cryosections were cut at −120°C, were transferred to ploioform-coated nickel grids, and were freeze-dried overnight. The sections were coated with a thin layer of carbon and viewed and analyzed in an FEI Tecnai 12 electron microscope equipped with an EDAX energy-dispersive x-ray spectroscopy detector. Mapping was achieved with EDAX software (EDAX) and used to display the distribution of gadolinium within the vessel wall.

### ICP-Mass Spectrometry for Gadolinium Quantification

To ensure the same pharmacokinetics/biodistribution of gadofosveset measured by T1 mapping in vivo and ICP-mass spectrometry (MS) ex vivo, the animals allocated for ICP-MS measurements were injected with gadofosveset after the post-trigger MRI and the agent was allowed to circulate in the body for 30 minutes. The animals were then euthanized, and aortas were collected as described above. For ICP-MS measurements aortic segments (n=14: 2 control, 6 stable, and 6 ruptured plaques) were digested in 70% nitric acid at 56°C for 15 to 18 hours and diluted with deionized water for ICP-MS analysis.

A standard curve was used for gadolinium concentration determination.

### Statistics

The Statistical Package for the Social Sciences 21.0 (IBM Corporation, Somers, NY) was used. For 2-group comparisons, continuous variables (eg, control versus diseased, control versus stable/ vulnerable, and stable versus vulnerable at the same time point) were compared using an unpaired t test. However, a paired t test was used to compare repeated measurements between 3 and 12 weeks (eg, R₁ at 3 and 12 weeks for the same animal). Correlation analysis was
performed using the Spearman test. Multiple segments from the same animal are assumed to be independent of each other. A second observer blinded to plaque status (stable/rupture-prone plaques) reanalyzed the R₁ maps to test the reproducibility of this measurement. The intraobserver and interobserver variability was assessed by using the interclass correlation coefficient (ICC) for continuous variables (R₁ values). The ability of MRI variables to detect rupture-prone plaque was assessed using sensitivity, specificity, positive and negative predictive values, and receiver-operator characteristic curve, and its area under the curve. Logistic regression analysis was used to identify predictors of rupture-prone plaque with the presence/absence of thrombus as the outcome variable. Continuous data are presented as the mean±SEM. Probability values of <0.05 were considered significant.

**Results**

**Vascular Permeability Is Higher in Rupture-Prone Than in Stable Plaque**

Consistent with previous studies, the 12-week pretrigger MRI showed aortic atherosclerosis in all animals and the post-trigger MRI showed plaque rupture in 6 of 10 (60%) animals characterized by mural thrombus. In a representative example, pretrigger T₁wBB images showed increased vessel wall area between 3 (Figure 1A and 1C) and 12 weeks (Figure 1E and 1G; 3.75±0.12 mm² versus 4.20±0.18 mm²; P=0.04), indicating atherosclerosis progression. However, the PA was similar between stable and rupture-prone plaques at both the 3 weeks (3.82±0.13 mm² versus 3.71±0.23 mm²; P=0.7) and 12 weeks (4.00±0.2 mm² versus 4.33±0.35 mm²; P=0.58). The vessel wall R₁ relaxation rate, measured 30 minutes after gadofosveset administration, was similar between stable and rupture-prone plaques at 3 weeks (Figure 1B and 1D; 1.50±0.12 versus 1.70±0.2 s⁻¹). Conversely, at 12 weeks, the rupture-prone plaque had significantly higher R₁ relaxation rate compared with the stable plaque, indicating higher vascular permeability (1.83±0.25 versus 2.54±0.3 s⁻¹; Figure 1F and 1H). Post-trigger T₁wBB images (Figure 1I and 1K) and corresponding histology (Figure 1J and 1 L) show the absence of thrombus in the case of the stable plaque and the presence of thrombus overlying the ruptured plaque. An additional example demonstrating R₁ and T₁ maps, both in gray-scale (Figure 1B, 1G, 1D, and 1I) and color-coded formats (Figure 1C, 1H, 1E, and 1J), in visualizing and segmenting the vessel wall to calculate the vessel wall T₁/R₁ values is illustrated in (Figure II in the Data Supplement). ICC analysis revealed high intra-rater agreement (ICC, 0.89; 95% confidence interval, 0.83–0.93; P<0.001) and good inter-rater variability (ICC, 0.75; 95% confidence interval, 0.4–0.79; P<0.001) for the measurement of vessel wall relaxation rate R₁. The results of the blinded observer are illustrated in Table I in the Data Supplement, alongside observer one.

**Quantitative MRI Measurements**

Quantification of vascular permeability to gadofosveset (R₁) during atherosclerosis progression in the rabbit aorta is illustrated in Figure 2. The vessel wall R₁ was similar in control and diseased animals at 3 weeks (1.69±0.02 versus 1.71±0.2 s⁻¹; P=0.19) but was significantly higher at 12 weeks (1.71±0.2 versus 2.00±0.1 s⁻¹; P<0.001; Figure 2A). When the vascular segments were classified into stable and rupture-prone plaques at 3 weeks (Figure 2B and 2D; 1.50±0.12 versus 1.70±0.2 s⁻¹). Conversely, at 12 weeks, the rupture-prone plaque had significantly higher R₁ relaxation rate compared with the stable plaque, indicating higher vascular permeability (1.83±0.25 versus 2.54±0.3 s⁻¹; Figure 2F and 2H). Post-trigger T₁wBB images (Figure 2I and 2K) and corresponding histology (Figure 2J and 2L) show the absence of thrombus in the case of the stable plaque and the presence of thrombus overlying the ruptured plaque. n=10 rabbits, 6 with ruptured and 4 without ruptured lesions were included in the MRI analyses. Ao indicates aorta; and V, vein.

![Figure 1](http://circimaging.ahajournals.org/)

**Figure 1.** In vivo magnetic resonance imaging (MRI) shows higher vascular permeability in rupture-prone compared with stable plaque in rabbit atherosclerosis. Native pretrigger T₁wBB images acquired at 3 wk (A and C) and 12 wk (E and G) show increased vessel wall thickening because of plaque formation. Corresponding R₁ relaxation maps (B, D, F, and H) show higher vessel wall relaxation rate (R₁; orange/yellow color; arrows) in rupture-prone (H) compared with the stable plaque (F) at 12 wk, indicating higher vascular leakage to gadofosveset. Post-trigger T₁wBB images (I and K) compared with the stable plaque (F) at 12 wk, indicating higher vascular leakage to gadofosveset. Post-trigger T₁wBB images (I and K) and histology (J and L) show the absence of thrombus at the site of a stable plaque and the presence of a thrombus overlying the ruptured plaque. n=10 rabbits, 6 with ruptured and 4 without ruptured lesions were included in the MRI analyses. Ao indicates aorta; and V, vein.
plaques, the vessel wall rate $R_1$ was similar between the groups at 3 weeks (1.70±0.17 versus 1.75±0.6 s⁻¹; $P = 0.33$). However, at 12 weeks, rupture-prone plaques had significantly higher gadofosveset uptake resulting in higher $R_1$ values compared with stable plaque (2.30±0.5 versus 1.86±0.3 s⁻¹; $P<0.001$). Paired $t$ test analysis showed a significant increase of $R_1$ between 3 and 12 weeks for both the stable (1.70±0.17 versus 1.86±0.3; $P<0.001$) and rupture-prone (1.75±0.6 versus 2.30±0.5; $P<0.001$) lesions (Figure 2B). To account for changes in $R_1$ that might be affected merely by lesion size, the $R_1$ was normalized to PA as measured by T1wBB images. The $R_1$/PA ratio was higher in rupture-prone than in stable plaque (0.70±0.06 versus 0.47±0.02; $P<0.001$) at 12 weeks (Figure 2C). Paired $t$ test analysis showed a small but significant increase in the $R_1$/PA ratio between 3 and 12 weeks for rupture-prone lesions (0.55±0.04 versus 0.70±0.06; $P=0.04$). The percentage increase in $R_1$ between 3 and 12 weeks was higher for rupture-prone than for stable plaque (50.80±7.2% versus 14.22±2.2%; $P<0.001$; Figure 2D). Gadolinium concentration was higher in diseased than in control animals (50.45±9.2 versus 22.13±1.0 μg/g tissue; $P=0.03$) and rupture-prone compared with stable plaque (80.54±6.13 versus 32.40±4.0 μg/g tissue; $P<0.001$) as calculated by ICP-MS.

**Vascular Permeability to Albumin Is a Predictor of Plaque Rupture**

The value of the quantitative assessment of vascular permeability ($R_1$) after administration of gadofosveset in detecting the rupture-prone plaque was analyzed with receiver-operator characteristic curve curves at 12 weeks (Figure 3A through 3C). Receiver-operator characteristic curves showed that $R_1$, $R_1$/PA, and the $\%AR_1$ between 3 and 12 weeks had high areas under the curve and were good predictors of rupture-prone plaque (Table). Logistic regression analysis showed that the $R_1$ at 12 weeks is a predictor of rupture-prone plaque ($P<0.001$; odds ratio, 11.45; confidence interval, 3.89–33.72).

**Histological Analysis Shows Higher Intra-Vessel Wall Albumin and Microvessel Density in Ruptured Compared With Stable Plaque**

Quantification of the albumin-immunopositive areas showed a significant increase in intravessel wall albumin in diseased compared with control vessels (Figure 4A through 4C [brown color] and Figure 4D; 33.00±3.3% versus 12.91±0.8%; $P = 0.01$) and particularly in ruptured compared with stable plaque (44.8±4.0% versus 25.93±3.0%; $P = 0.03$; Figure 4E). CD31-staining of the endothelium (Figure 4F through 4H, brown color) showed intact, continuous ECs in control vessel, less structured cells in stable plaque, and fragmented cells covering the lumen of ruptured plaque. Endothelium staining also showed higher microvessel density in ruptured than in stable plaque (21.00±2.24 versus 7.32±0.96; $P<0.001$) and the absence of microvessels in control arteries (Figure 4I through 4K and 4 L).

**TEM Shows Abnormal Morphology and Aberrant Junctions of Luminal and Microvascular ECs**

TEM was used to examine the ultrastructural integrity of luminal and microvascular ECs. Control luminal endothelium was characterized by intact basal lamina (BL) and interendothelial junctions indicated by close contacts (Figure 5A). However in diseased arteries, endothelial integrity was severely compromised. The luminal endothelium appeared detached from the BL (Figure 5B) with numerous intracytoplasmic vacuoles (Figure 5C). The interendothelial contact was incomplete or completely absent allowing infiltration of blood erythrocytes within the vessel wall (Figure 5C). In more advanced cases, the luminal endothelium showed signs of cellular death including lack of nucleus and denudated regions allowing exposure of the subendothelial space (Figure 5D). Similar to the luminal endothelium, microvascular endothelium was also structurally compromised (Figure 5E through 5H). In some cases, microvascular ECs showed intact BL and closed interendothelial junctions (Figure 5E). However in other cases, microvascular...

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**Figure 2.** Quantification of magnetic resonance imaging (MRI) measurements after administration of gadofosveset. A and B, Vessel wall $R_1$ increased with disease progression and is higher in rupture-prone than in stable plaque at 12 wk. C, $R_1$/plaque area was higher in rupture-prone than in stable plaque at 12 wk. D, The percentage change in vessel wall $R_1$ between 3 and 12 wk was significantly higher in rupture-prone than in stable plaque. n=10 rabbits, 6 with ruptured and 4 without ruptured lesions were included in the MRI analyses.
ECs showed various structural changes (Figures 5F through 5H). Although an intact BL was generally observed, interendothelial contact was incomplete (Figure 5G, dashed allows) and endothelium morphology was changed to a more cuboidal shape with numerous intracytoplasmic vacuoles (Figure 5G, arrows). Detachment of ECs from the BL and leucocytes adhering to the microvessel lumen and infiltrating into the plaque was also observed (Figure 5H).

TEM and gadolinium mapping using x-ray spectra showed a diffuse distribution of gadofosveset across the vessel wall and lack of colocalization to particular plaque or vessel wall components (Figure III in the Data Supplement).

There were significant correlations between vascular permeability (measured by $R_1$) and PA (measured by $T_1wPA$) at 3 ($r=0.23; P=0.03$) and 12 ($r=0.24; P=0.03$) weeks (Figure IV A and IVB in the Data Supplement). However, when the analysis was repeated with vascular segments classified as stable or rupture-prone plaque, the correlation was more significant for stable ($r=0.4; P=0.004$) but disappear for rupture-prone ($r=0.2; P=0.44$) lesions at 12 weeks (Figure IVC and IVD in the Data Supplement). We also found that the percentage increase in R1 was not followed by an equivalent increase in PA (Figure VA in the Data Supplement). The percentage increase in vascular permeability ($\Delta R_1$) to gadofosveset between 3 and 12 weeks was significantly higher for rupture-prone than for stable plaque (50.80±7.2% versus 14.22±2.2%; $P<0.001$). However, the percentage increase of PA ($\Delta T_1wPA$) was not statistically different between the 2 lesion types (35.00±3.5% versus 41.00±9.0%; $P=0.2$). Furthermore, there was lack of correlation between the percentage change of PA ($\Delta T_1wPA$) and the percentage change in vascular permeability ($\Delta R_1$) between 3 and 12 weeks (Figure VB in the Data Supplement) when all lesions were included ($r=0.16; P=0.8$). However, there was a significant correlation between $\% \Delta T_1wPA$ and $\% \Delta R_1$ for stable ($r=0.32; P=0.02$), but not for rupture-prone lesions ($r=-0.08; P=0.48$; Figure VC and VD in the Data Supplement).

**Intrinsic Vascular Elasticity Decreases in Diseased Vessels and Is Lower in Rupture-Prone Than in Stable Plaque**

Functional changes in the intrinsic vasodilation and wall stiffness were also investigated. The intrinsic vasodilation was lower in atherosclerotic than in control animals (control, 26.80±1.72% versus 3 weeks, 23.43±0.8%; $P=0.07$; control versus 12 weeks, 18.60±1.0%; $P<0.001$; Figure 6A). Vasodilation was even lower in rupture-prone than in stable lesions at both the 3-week (21.21±1.3% versus 24.41±1.13%; $P=0.02$) and 12-week (16.40±2.0% versus 21.63±1.2%; $P<0.001$) time points (Figure 6B). However, there was lack of correlation between the intrinsic vasodilation and PA at both the 3 weeks ($r=-0.02; P=0.45$) and 12 weeks ($r=-0.02; P=0.5$, data not shown).

**Table. Ability of Magnetic Resonance Imaging Measurements in Detecting Rupture-Prone Plaques**

<table>
<thead>
<tr>
<th></th>
<th>$R_1 \geq 1.9$ s$^{-1}$ and % (CI)</th>
<th>$R_1/T_1wPA \geq 0.3938$, s$^{-1}$/mm$^2$, and % (CI)</th>
<th>$\Delta R_1 \geq 24.81%$, and % (CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity</td>
<td>81.2 (63.8–92.1)</td>
<td>90.6 (73.8–97.5)</td>
<td>65.6 (48.1–81.4)</td>
</tr>
<tr>
<td>Specificity</td>
<td>72.5 (58.0–83.6)</td>
<td>45.0 (91.8–59.5)</td>
<td>84.3 (71.4–92.9)</td>
</tr>
<tr>
<td>PPV</td>
<td>48.2 (37.2–59.3)</td>
<td>68.7 (57.4–78.2)</td>
<td>72.4 (52.8–87.3)</td>
</tr>
<tr>
<td>NPV</td>
<td>51.8 (40.0–62.8)</td>
<td>31.3 (21.8–42.6)</td>
<td>79.6 (66.5–89.4)</td>
</tr>
</tbody>
</table>

CI indicates confidence interval; NPV, negative predictive value; and PPV, positive predictive value.
Aortic stiffness measured with PWV increased in diseased aortas at 12 weeks compared with 3 weeks in control animals (5.00±0.1, 2.50±0.2, and 2.53±0.2 m/s; Figure 6C). Paired t test showed a significant increase in PWV between 3 and 12 weeks in both animals with stable (2.49±0.3 versus 5.23±0.4 m/s; \( P=0.03 \)) and animals with rupture-prone (2.49±0.1 versus 5.00±0.04 m/s; \( P<0.001 \)) lesions. However, PWV was not statistically different between animals with stable and rupture-prone plaques at each time point (2.49±0.3 versus 2.49±0.1 m/s; \( P=1.0 \) and 5.23±0.35 versus 5.00±0.03 m/s; \( P=0.55 \); Figure 6D). There was no correlation between PWV and PA at 3 weeks (\( r=0.54; P=0.2 \)), but there was a significant correlation at 12 weeks (\( r=0.9; P=0.037 \); Figure 6E), suggesting that lesion expansion increases arterial stiffening. Finally, there was a significant negative correlation between the percentage changes in the intrinsic vasodilation and arterial stiffness (\( r=-0.96; P=0.003 \); Figure 6F).

Discussion

This study demonstrates that vascular permeability, measured using an MR albumin-binding contrast agent and T1 mapping, is elevated in atherosclerotic compared with control vessels and, more importantly, is higher in rupture-prone than in stable atherosclerotic lesions in a rabbit model of the disease. Increased vascular permeability measured in vivo was histologically validated by increased intravessel wall albumin, higher microvessel density, and lack of structural integrity of both the luminal and microvascular endothelium. Functionally, the intrinsic capacity of the vessel wall to vasodilate was significantly lower in diseased, particularly, in rupture-prone lesions. Furthermore, PWV analysis revealed significantly increased arterial stiffness in atherosclerotic compared with control animals, but had similar levels of stiffness between animals that had stable and rupture-prone lesions. We showed that MRI can quantify both morphological and functional changes of the vessel wall and that noninvasive quantification of vascular permeability using an albumin-binding MR contrast agent may have diagnostic value in detecting not only plaque progression but also unstable atherosclerotic lesions.

Traditionally, bright-blood dynamic contrast enhanced-MRI has been used to study plaque permeability. Measurements demonstrated that the kinetic parameters including fractional plasma volume (vp) and \( K_{\text{trans}} \) (expressing flow/permeability) correlate with microvessel density,\(^{20-23}\) adventitial vaso-vasora,\(^{24,25}\) and with sites of active inflammation.\(^{26}\) \( K_{\text{trans}} \) was also used to monitor response to therapeutic intervention.\(^{37}\) Human subjects treated with lipid-lowering therapy for 1 year showed reduced \( K_{\text{trans}} \) independently of a reduction in the lipid-rich necrotic core size or circulating levels of C-reactive protein.\(^{37}\) Despite the promising applications
of bright-blood dynamic contrast enhanced-MRI, acquisitions are usually suited for the characterization of lesions thicker than 2 mm because of the close proximity of the enhancing vessel lumen, which makes vessel wall segmentation challenging. Alternatively, contrast uptake can be evaluated using black-blood dynamic contrast enhanced-MRI by measuring the area under the curve. Experiments in atherosclerotic rabbits showed a correlation between area under the curve, after administration of Gd-DTPA and histological markers of plaque neovascularization.

Our findings using a T1 mapping approach showed that the vessel wall relaxation rate (R₁) after gadofosveset administration could also be used to quantify changes in vascular permeability in the context of atherosclerosis. We found that vessel wall permeability, measured as R₁, was significantly higher at 12 than at 3 weeks after commencing the experimental protocol and control animals. Importantly, rupture-prone plaques had significantly higher R₁ values and R₁/PA ratio compared with stable lesions at 12 weeks. The percent change in R₁ between 3 and 12 weeks was also significantly higher for rupture-prone than for stable lesions. We have previously shown that vessel wall uptake of the albumin-binding contrast agent associates not only with the plaque progression, as we have previously shown, but also with the lesion instability using a rabbit model that replicates multiple histological features described for human plaques. The ability to experimentally induce plaque rupture and thrombosis at a precise time point provides a functional end point to retrospectively classify plaque as nonruptured/stable (no thrombus) or ruptured/vulnerable (mural thrombus). Therefore, this study protocol allows determination of imaging measurements that could potentially predict complication that cannot be achieved in the murine models we have previously used. The clinical utility of gadofosveset for vessel wall imaging was shown in a small proof-of-principle study that showed significantly higher gadofosveset uptake in symptomatic compared with asymptomatic human carotid plaques. Our current results are in line with human data and pinpoint the potential value of quantitative measurement of vascular permeability using the albumin-binding contrast agent for identification of high-risk/ unstable plaques.

Histologically we found increased intravessel wall albumin and microvessel density in diseased and in rupture plaques in particular. TEM analysis revealed loss of the endothelial integrity with disruption of interendothelial junctions in both the luminal and microvascular endothelium of atherosclerotic plaques. Both luminal and microvascular ECs seemed detached from the BL and had a cuboidal shape and numerous intracytoplasmic vacuoles, a sign of increased secretory capacity. Interendothelial contact was incomplete or absent allowing infiltration of blood cells (erythrocytes and leukocytes adhering to the microvessel lumen thus infiltrating the plaque were also observed). n=8; 2 control, 3 stable, and 3 ruptured plaques were included. RBC indicates red blood cell.
leucocytes) into the vessel wall. Studies in humans showed that rupture-prone and ruptured coronary plaques exhibit a 2- and 4-fold increase in microvessel density, respectively, compared with stable obstructive plaques. Similarly, ruptured carotid endarterectomy and aortic plaques had increased microvessel density with larger, immature, more irregular-shaped microvessels comparable to those found in tumors and healing wounds. Thin-walled microvessels showing incomplete endothelial junctions and lack of structural integrity have also been reported in human coronary plaques and were used to explain the extensive leucocyte infiltration, intraplaque hemorrhage, and plaque instability. Our histological data corroborate previous observations and suggest that increased microvessel density and compromised structural integrity (of both the luminal and microvascular endothelium) increase vascular leakage that facilitates extravasation of inflammatory cells and molecules that may contribute to plaque instability. Our in vivo measurements further validate ex vivo histological findings and provide a quantitative method for in situ measurement of focal vascular leakage associated with atherosclerosis progression and plaque instability using the albumin-binding contrast agent as a surrogate marker.

Our study also revealed some in vivo mechanistic insights on the role of vascular leakage in the natural evolution of atherosclerosis and plaque instability. Although we found that vascular permeability was increased in diseased compared with control arteries and in rupture-prone compared with stable lesions, the percentage increase in vascular permeability (%ΔR1) was not followed by an equivalent increase in PA (%ΔT1wPA) between 3 and 12 weeks when all arterial segments were analyzed together. However, when stable and rupture-prone lesions were analyzed independently, a significant correlation between %ΔT1wPA and %ΔR1 was found for stable lesions. These data suggest that the rate of plaque growth may be affected by changes in vascular permeability at earlier stages of disease progression. We speculate that at some point...
during plaque evolution increased vascular leakage because of (1) increased microvessel density, (2) differential permeability of individual microvessels, or (3) lack of structural integrity of the luminal or microvascular endothelium precedes plaque expansion causing a divergence from linearity between the increase of vascular permeability and focal lesion. To this end, measuring vascular permeability may provide a more sensitive biomarker for assessing atherosclerotic risk.

This study has also revealed functional changes (vasodilation and stiffness) in the vessel wall in atherosclerosis, in particular, in relation to the properties of stable compared with rupture-prone plaque. Intrinsic vasodilation decreased with the progression of atherosclerosis and was lower in rupture-prone than in stable lesions. However, there was no significant correlation between the intrinsic vasodilation and PA, suggesting that factors other than plaque size may contribute to the reduced vasodilation. Other studies have shown that structural changes in the vascular endothelium reduce endothelium-dependent vasodilation in response to endothelium stressors, or mechanoreceptor signaling. EC surfaces have multiple mechanoreceptors that sense and respond to changes in endothelial shear stress (ESS) via intracellular mechanotransduction pathways. Physiological pulsatile ESS regulates continuous nitric oxide (NO) production by the endothelium either at the transcriptional level, through upregulation of endothelial nitric oxide synthase (eNOS) gene expression, or at the post-transcriptional level by eNOS protein phosphorylation and activation. NO is a potent regulator of healthy vascular tone. Low ESS reduces the bioavailability of NO by decreasing eNOS mRNA and protein expression, downregulates prostacyclin, an endothelial vasodilatory substance, and upregulates endothelin-1, a vasoconstrictive molecule. Although we did not measure ESS in this study, our previous work using this animal model demonstrated that low ESS was associated with plaque burden, positive vascular remodeling, and plaque instability. Similar results were shown using other animal models and in humans and could probably explain the reduction in the intrinsic vasodilation. Our study also showed coupling between morphological (PA) and functional (stiffness) vascular changes. PWV did not increase in the early stages of atherosclerosis (3 weeks), but increased after 12 weeks and correlated with PA. However, the increase in PWV was similar in animals with and without rupture-prone plaques. Our data suggest that vascular stiffening maybe a measure of overall vascular health (when atherosclerotic lesions are well formed), but it is not a surrogate marker of the propensity of a plaque to rupture in this animal model. Similar data have been reported in human studies where PWV was shown to increase with age, in the presence of calcification, hypertension, and intraplaque hemorrhage.

**Limitations**

A potential limitation of our study is that noncontrast T1wBB (0.31x0.31 mm) had a higher in-plane resolution compared with the T1 mapping (0.4 x 0.4 mm) and that partial volume effects and motion might influence the accuracy of the R1 measurements. However, the thickness of the normal vessel wall in the rabbit aorta is ~0.5 to 0.6 mm and therefore in-plane resolution should have been sufficient to delineate the vessel wall on both sequences.

The higher ICC observed for the intra-reader variability than for the inter-reader variability is not surprising and suggested a higher variability between independent observers when manually segmenting the vessel wall for measuring the R1 when compared with the same observer. Manual vessel wall segmentation is known to be both time consuming and also subject to observer variability. In our study, the R1/T1 maps were analyzed using a dedicated Matlab code that could only load the T1 mapping data sets. Fusion of the R/T maps with the corresponding black blood images that could potentially facilitate vessel wall segmentation was not possible. Future adaptations of our code might allow for this function. In addition, the future use of 3D Modified Look-Locker inversion recovery-based T1 mapping sequences that allow for immediate reconstruction of the T1/R1 maps that can be directly imported on the Digital Imaging and Communications in Medicine viewer along with the black-blood images, perform image fusion, and coregistration might also improve the accuracy and decrease inter-rater the variability in vessel wall segmentation.

**Conclusions**

Compromised structural integrity of the luminal and microvascular endothelium and higher microvessel density increase vascular permeability in atherosclerosis and particularly rupture-prone plaque. T1 mapping using an albumin-binding contrast agent could be used to quantify changes of vascular permeability associated with atherosclerosis and plaque instability.

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**Disclosures**

None.

**References**


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21. Phinikaridou et al. MRI of Plaque Permeability and Instability


Compromised structural integrity, of both the luminal and microvascular endothelium, and higher microvessel density are well-documented features of atherosclerotic lesions and, particularly, ruptured atherosclerotic lesions. Such structural changes collectively increase vascular permeability that accompanies the development and severity of the disease. However, noninvasive imaging of endothelial function and vascular permeability has only recently been demonstrated by magnetic resonance imaging. Quantitative assessment of albumin leakage into the vessel wall using an albumin-binding contrast agent and T1 mapping protocols may provide a methodology for monitoring not only atherosclerotic lesion progression but also detecting rupture-prone lesion before a clinical complication. The fact that the albumin-binding agent is clinically approved allows for translation of this imaging approach in humans with established atherosclerotic disease to determine imaging measurements that could predict lesion instability.
Increased Vascular Permeability Measured With an Albumin-Binding Magnetic Resonance Contrast Agent Is a Surrogate Marker of Rupture-Prone Atherosclerotic Plaque

Alkystis Phinikaridou, Marcelo E. Andia, Begoña Lavin, Alberto Smith, Prakash Saha and René M. Botnar

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**SUUPLEMENTAL MATERIAL**

**Supplemental Methods**

**Experimental Protocol**

- Endothelial denudation of the abdominal aorta
- **High cholesterol diet** vs. **Normal diet**

**Experimental Protocol**

- **Day 1 Pre-trigger MRI**
  - **0h**
- **Day 1 Triggering**
  - **4h**
- **Day 2 Post-trigger MRI**
  - **1st and 2nd trigger for plaque rupture**

**Figure 1**: Experimental protocol, timings of the MRI scans and acquisition sequences.

**MR Image analysis of R1/T1 maps**

The T1 mapping datasets were imported in a Matlab code, developed in-house. For every slice, 16 T1 weighted images with different inversion delays were acquired. The Matlab software could then compute the R1 and T1 maps on a pixel-by-pixel basis using a 3-parameter curve fitting of the longitudinal magnetization $M_z(T_1)$ including a $T_1$ correction based on the following formulas:

$$M_z(T_1) = A - Be^{(T_1/T_1')},$$

with $A = M_0^*$ and $B = M_0 + M_e^*$. Subsequently, $T_1$ can be estimated directly from the fit parameters: $T_1 = T_1^* (B/A - 1)$

$M_0^*$ is the equilibrium magnetization. As the longitudinal magnetization is sampled during its recovery, the relaxation process is influenced by the constant application of RF pulses resulting in the measurement of an apparent relaxation time: $T_1^* (T_1^*<T_1; R_1=1/T_1; 1/T_1^* = 1/T_1 - (1/\text{TR}) \ln(\cos))$ and a reduced equilibrium magnetization $M_e^*$. After placing the ROI, the software computed only the mean $R_1$ values of the pixels included within the ROI.
Additional Figures

**Figure 2: Vessel wall $R_1$ and $T_1$ maps**

Examples of vessel wall T1wBB, $R_1$ and $T_1$ maps in lower (A-E) and higher resolution (F-J) used for vessel wall visualization and segmentation for calculating the corresponding $R_1$ values. Maps are shown both as grey-scale (B, G, D I) and color-coded (C, H, E, J). N=10 rabbits, 6 with ruptured and 4 without ruptured lesions were included.
Supplemental Table 1

<table>
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<tr>
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<th>Observer 1</th>
<th>Observer 2</th>
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<td>Diseased 3 weeks</td>
<td>Diseased 12 weeks</td>
<td>( P ) values</td>
<td>Diseased 3 weeks</td>
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<td>( R_1 ) (s(^{-1}))</td>
<td>1.71±0.2</td>
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<td>Rupture-prone</td>
<td>Stable</td>
<td>Rupture-prone</td>
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<tr>
<td>( R_1 ) (s(^{-1}))</td>
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<td>3weeks</td>
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<td>2.63±0.13</td>
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<td>12weeks</td>
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<td></td>
<td></td>
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<tr>
<td>% change ( R_1 )</td>
<td>14.22±2.2</td>
<td>50.80±7.2</td>
<td>&lt;0.001</td>
<td>32.90±4.5</td>
</tr>
</tbody>
</table>

The cut off values and ROC curve statistics for detecting rupture-prone plaque using the measurements of Observer 2:

**Sensitivity \( R_1 \) (s\(^{-1}\)):** cut off value \( \geq 2.9\)s\(^{-1}\), AUC= 0.74, 95% CI =0.62-0.87, \( P=0.002 \)

**Sensitivity \( \Delta R_1 \) (s\(^{-1}\)):** cut off value \( \geq 88.57 \), AUC= 0.79, 95% CI =0.65-0.93, \( P<0.001 \)

N=10 rabbits, 6 with ruptured and 4 without ruptured lesions were included.
Figure 3: Gadofosveset distribution within the vessel wall.

Transmission electron microscopy and gadolinium mapping using x-ray spectra showed a diffused distribution of gadofosveset across the vessel wall and lack of co-localization to particular plaque or vessel wall components. Aortic segments (n=2; stable plaque) were used for this analysis.
Figure 4: Correlation analysis between plaque area and vascular permeability. (A-B) There was a significant linear correlation between vascular permeability, as measured in vivo by quantification of the R1, 30min post gadofosveset administration, and plaque area as measured by T1wBB MRI for both time points. (C-D) The linear correlation was even stronger and more significant for the stable but disappear for the rupture-prone lesions at 12 weeks. N=10 rabbits, 6 with ruptured and 4 without ruptured lesions were included.
Figure 5: Correlation analysis between the percentage of change of plaque area and vascular permeability.

(A) The percentage increase in R1 was not followed by an equivalent increase in plaque area (T1wBB images). The percentage increase in vascular permeability to gadofosveset (% ΔR₁) between 3 and 12-weeks was significantly higher for ruptured compared for stable plaque. However, the percentage increase of plaque area (%ΔT₁wPA) was not significantly different between stable and rupture-prone plaque. (B) There was lack of correlation between the percentage plaque growth (%ΔT₁wPA) and the percentage change in vascular permeability (%ΔR₁) between 3 and 12-weeks when all lesions were included. (C-D) There was a significant linear correlation between %ΔT₁wPA and %ΔR₁ for stable compared to rupture-prone lesions. N=10 rabbits, 6 with ruptured and 4 without ruptured lesions were included.