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ARTICLE

Short Term Arterial Remodelling in the Aortae of Cholesterol Fed New Zealand White Rabbits Shown in vivo by High-Resolution Magnetic Resonance Imaging - Implications for Human Pathology*

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High-resolution, non-invasive imaging methods are required to monitor progression and regression of atherosclerotic plaques. We investigated the use of MRI to measure changes in plaque volume and vessel remodelling during progression and regression of atherosclerosis in New Zealand White rabbits. Atherosclerotic lesions were induced in the abdominal aorta by balloon injury and cholesterol feeding. MR images (2D) of the abdominal aorta were acquired with cardiac and respiratory gating using a fast spin echo sequence with and without fat-suppression. In an initial study on rabbits treated for 30 weeks we imaged the aortae with a spatial resolution of 250x250 micrometers with a slice thickness of 2 mm and achieved a close correlation between MRI-derived measurements and those made on perfusion pressure-fixed histological sections (r_1 = 0.83, slope p_1 < 0.01). We subsequently imaged 18 rabbits before and periodically during 12 weeks of cholesterol feeding (progression) followed by 12 weeks on normal diet (regression). Aortic wall (atherosclerotic lesion) volume increased significantly during progression and decreased during regression. In contrast, lumen volume increased during progression and did not change during regression. In conclusion, this study confirms that non-invasive, high-resolution MRI can be used to monitor progression and regression of atherosclerosis, each within 3 months and shows, for the first time in a short-term model, that positive remodelling occurs early during progression and persists through regression of atherosclerotic lesions. (Pathology Oncology Research Vol 10, No 3, 159–165)

Keywords: animal models of human disease, imaging, MRI, atherosclerosis, remodelling

Introduction

Various new imaging modalities including magnetic resonance imaging (MRI) have become the subject of intensive investigation in histopathology[1,2,3] and forensic medicine.[4,5] They are used to assist[2,3,4] and, under certain circumstances, to replace traditional methods of investigation.[4,5] We have examined the power of high-resolution non-invasive MRI to image components of plaques in the aortae of an animal model of atherosclerosis. We show some of the benefits of MRI over traditional histopathological methods as well as report certain limitations of the technique. One of the major benefits of MRI is that it is possible to use in vivo, which allows dynamic changes in pathological conditions to be observed without repeated sampling of tissues. This is most important in cases, in which the nature of the disease is focal or taking a tissue sample would require major surgical intervention, such as in coronary artery disease.

The current "gold standard" for identifying and monitoring atherosclerosis in humans is contrast angiography

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which measures the stenosis of the arterial lumen. However, arteries can compensate for the progressive growth of atherosclerotic plaques by increasing their external circumference thereby maintaining lumen diameter, a process called outward or positive remodelling.[6] Angiography, therefore, fails to identify non-stenotic lesions in remodelled vessels. In addition, because of both positive and negative remodelling, changes in plaque volume induced by medical therapy may not be reflected in changes in lumen stenosis. Furthermore, because of the invasive nature of angiography and the radiation involved, it cannot easily be used to monitor plaque development. Traditional histopathological methods are also not suitable for this purpose. There is a need for novel, non-invasive methods for quantifying plaque volume and, ideally, composition independently of lumenal stenosis. MRI has previously been used to identify the structure of atherosclerotic plaques both in animal models[7,8] and in humans.[9] Long-term remodelling of aortae in Watanabe heritable hyperlipidemic rabbits has also been demonstrated by serial MRI.[10] The purpose of the present study was, firstly to optimise the resolution of the MRI equipment to identify plaque composition in established atherosclerotic lesions and, secondly to undertake a detailed study of the effects of atherosclerotic plaque progression and regression on vessel remodelling.

Methods

All experiments complied with the UK Animals (Scientific Procedures) Act 1986 and the GlaxoSmithKline Code of Practice for Animal Experimentation.

Study 1 protocol

Female New Zealand white (NZW) rabbits (Charles River, n = 5, 10 week old) were fed rabbit maintenance (RM) diet (SDS, Witham, UK) supplemented with 0.2% cholesterol[11] and with reduced vitamin E (25 mg/kg) for a total of 30 weeks. Two to 4 weeks after commencement of the modified diet, the rabbits were subjected to balloon-induced intimal injury of their abdominal aortae (see below). The rabbits were imaged once at 25-30 weeks after starting the atherogenic diet and then culled to remove tissue for histology a few days after imaging.

Study 2 protocol

Eighteen 10 week old female New Zealand white (NZW) rabbits (Charles River) were fed normal 2031 diet (Harlan Teklad, UK) containing 0.2% added cholesterol and with or without 5% peanut oil (n = 9/group) for 12 weeks before returning to normal diet for a further 12 weeks. Rabbits were imaged immediately after starting the diet and at 8, 12, 16, 21 and 24-25 weeks and then culled to remove tissue for histology.

Balloon angioplasty

Atheroma was induced in the abdominal aorta by a combination of a high cholesterol diet and balloon-induced intimal injury. Two to 4 weeks after starting on high-cholesterol diet all the rabbits were premedicated with fentanyl 0.02 mg/kg and fluanisone ("Hypnorm" Jannsen, High Wycombe, Bucks, UK) 1 mg/kg intramuscularly (i.m). Once sedated 6 mg/kg propofol ("Rapinovet", AstraZeneca, UK) was administered intravenously (i.v.) via a marginal ear vein. The rabbits were then intubated with a 3.5mm uncuffed endotracheal tube (Portex, UK) under direct visualization of the larynx using a Millar size 1 laryngoscope blade, adapted to provide supplemental oxygen, and then supplied with isoflurane in oxygen at a concentration of approximately 1.5-2.5% as required. Animals breathed spontaneously throughout. The cardiovascular system was monitored by a pulse oximeter applied to the ear. The femoral artery was exposed, ligated distally with 3 metric braided nylon ("Neurolon" Ethicon, UK) and 0.2 ml of a 5% solution of papaverine (Sigma, UK) was applied to the surface of the vessel to facilitate passing of a 4F Fogarty embolectomy catheter into the aorta, to the level of diaphragm. The balloon was then inflated to a pressure of 1 atmosphere, and withdrawn until the aortic bifurcation was reached. Then the catheter was removed and the femoral artery ligated proximally. The wound was then closed with a subcutaneous polyglactin suture ("Vicryl", Ethicon, UK). Enrofloxacin ("Baytril") at 10 mg/kg and buprenorphine ("Vetergesic") 0.01 mg/kg were administered subcutaneously at cessation of surgery, with a further dose of buprenorphine (0.01 mg/kg) the following morning. Rabbits were checked daily for the duration of the study.

Plasma lipids

Plasma cholesterol and triglyceride levels were measured as described previously[12] using 1ml of blood taken into K EDTA tubes at approximately 4 week intervals.

All values were expressed as mean±SD.

MRI of atherosclerotic lesions

Images were acquired using a 2T Bruker Medspec Avance scanner with a 63 cm whole-body transmitter and a 20 cm birdcage resonator as receiver. In each imaging session a respiratory- and ECG-gated 2D Fast Spin Echo scan (RARE factor 4) with inflow saturation (NFS) was acquired. For all the imaging sessions an additional Fast

Spin Echo scan with fat suppression (FS – bandwidth 1kHz) was acquired. For each scan, ten 2 mm thick slices were acquired at end-expiration covering the abdominal aorta over the region of the renal arteries and extending distally beyond the inferior renal artery. The data matrix was 512^2 (in-plane resolution 250 µm) and TE_{eff} 39 ms.

Histological methods

Rabbits were culled by an overdose of sodium pentobarbitone (Euthatal, Rhone Merieux), perfused with oxygenated phosphate buffered saline (PBS) at 37ºC and physiological pressure followed by either 4% buffered formaldehyde or 4% glutaraldehyde (approximately 81 mm Hg, 30 minutes each).

MR images and histological sections were matched using a similar method to that validated by Worthley et al.[10] After perfusion, aortae were carefully removed from the arch to the iliac bifurcation, along with the heart and both kidneys, and placed into PBS. The heart, kidneys and iliac bifurcation served as important landmarks to orientate the aorta including the segment of abdominal aorta imaged by MRI. The region of abdominal aorta imaged by MRI as described above was excised, cut into 2 mm long segments and formaldehyde perfused tissue embedded in paraffin wax and glutaraldehyde perfused tissue in epoxy resin ready for histology. Individual MRI slices were matched with histological blocks of aorta by measuring the distance from the renal arteries. The 2 mm long histological segments, matching the corresponding MR images were serially sectioned, care being taken to monitor the orientation of the sections throughout. At least three sections from each segment were image-analysed as described below. Paraffin sections (5 µm) were stained with haematoxylin and eosin and also used for immunohistochemistry, while resin sections (1 µm) were stained with methylene blue and used to achieve the best possible histological morphology with minimal shrinkage. Due to low sample numbers, however, formal comparison between wax and resin sections was not undertaken. The data was pooled on the basis of the means appearing to be of similar magnitude. Images of the aortic cross-sections were captured digitally and analysed by computer assisted image analysis as described below.

Immunohistochemistry

Immunohistochemistry was performed on paraffin wax sections. After antigen retrieval by microwave irradiation (pH 6.0), non-specific staining was blocked by serum pretreatment, and the sections were then incubated with mouse monoclonal antibodies; anti – rabbit macrophage (RAM11, DAKO) and anti-smooth muscle actin (ICN Biomedicals Inc., Costa Mesa, California, USA). Sections were then treated with biotinylated goat anti – mouse immunoglobulins (Ig) antibody (DAKO) followed by incubation with avidin-alkaline phosphatase complex. Binding sites were visualized using a Vector Red substrate kit (Vector Laboratories). For a negative control the primary antisera were replaced with mouse immunoglobulins to assess non-specific binding.

Image analysis

Study 1. A total of 23 FS MR images of the aortae of 5 rabbits were analysed by computerized planimetry using an image analysis software package (Optimas 6.5) running on a PC. Anatomical regions of the retroperitoneum, which included the aorta, were selected by manual tracing, then within this zone pixels forming the aorta were chosen by computerized thresholding based on greyscale intensity. Measurements were of the cross-sectional area of the entire aortic wall on individual MRI slices. Digitised images of paraffin wax and resin embedded histological sections were analysed using the same software package to determine cross-sectional areas of intima plus media. All values were expressed in mm^2. The values obtained from the MR images were correlated with the average lesion (wall) area measured in three histological sections from the corresponding segment of aorta using linear regression analysis.

Study 2. FS and NFS images from 15 animals in study 2 were analyzed both by the method used in study 1 to confirm the correlation between lesion (wall) areas measured by MRI and histology, and by manual segmentation using ParaVision (Bruker) software. In the latter, the total area of the wall+lumen was determined for each of the ten slices of each of the animals at all 5 timepoints by tracking the exterior edge of the aorta (this was aided by the FS images) and then the area of the lumen was determined by tracing around the lumen of the aorta. Subtraction yielded the area of the vessel wall, which included the lesions. Multiplication by the slice thickness gave volumes of wall and lumen in each slice. Summation of the wall and lumen volumes of all slices gave the total lumen and wall volumes in each imaged aorta section. Volume was expressed in cm^3. A total of 300 MR images (150 FS and 150 NFS images) were used to obtain the results of serial imaging of which 27 randomly selected FS images were used to establish correlation between wall areas measured by MRI and histology as described above.

Statistical analysis

For study 1, comparison between cross-sectional areas of aortae measured by MRI and histology was made by linear regression analysis. Correlation was regarded as significant when $p<0.05$ for the slope of the regression

line. For study 2, firstly linear regression analysis was performed as described in study 1. Secondly, lumen and wall volumes were log transformed and analysed separately using a repeated measures ANOVA with bodyweight used as a covariate. The mean ratio to baseline at each imaging week was estimated from the ANOVA along with the 95% confidence intervals. Significance of ratios was tested using the Least Significant Difference test.

Results

Study 1

All 5 rabbits in study 1 survived to the end of the study. Plasma cholesterol rose from 1.7 ± 0.6 mmol/l to 27.1 ± 6.6 mmol/l ($p<0.001$) on feeding high-cholesterol diet and triglyceride levels did not change. Histological examination showed all rabbits developed extensive intimal thickening in their abdominal aortae (*Figure 1*). Immunohistochemistry showed that the composition of atherosclerotic lesions was similar to that of atheroma in humans[6,8,13] comprising a well-developed smooth muscle cell-rich fibrous cap overlying a macrophage-rich lipid core (*Figure 1*). The high in-plane resolution afforded by MRI of 250 µm enabled lesions as small as 300 µm in thickness to be visualised. FS MR images were available from all rabbits. Aortae from two of the rabbits were resin embedded whilst tissue from the remaining three rabbits was embedded into paraffin wax. Cross-sectional areas of aortic wall measured by MRI correlated well with cross-sectional areas of aortic intima plus media measured histologically (r_1 = 0.83, slope $p_1<0.01$) (*Figure 2*). FS MRI showed areas of signal hypointensity and signal voids within the lesions. These areas were shown by histology to correspond with calcification, macrophage-lipid-rich zones or regions devoid of intimal thickening. However, the FS and NFS MR images alone could not distinguish between these different morphologies (*Figure 3*).

Study 2

Fifteen of the 18 rabbits in study 2 survived to the end of the study. MRI showed an increased volume of aortic wall over the 12-weeks of cholesterol feeding. Wall volume over the entire imaged aorta increased by 151% (CI; 93%-226%, p = 0.0001) from 0.116 cm^3 to 0.292 cm^3. By week 24, 12 weeks after return to normal diet, vessel wall volume had reduced by 40% (CI; 28-50%, p = 0.0001 compared with week 12) *(Figure 4),* by which time wall volume had returned almost to pre-cholesterol diet-feeding levels (0.175 cm^3, CI; 0.141-0.216 cm^3). Lumen volume increased significantly over the first 8 weeks (p=0.0008), and then remained unchanged throughout the rest of the study (*Figure 4*). There was no difference in either the aortic wall or lumen volumes between the groups fed 0.2% cholesterol only or 0.2% cholesterol + 5% peanut oil.

Histological sections of the aortae were obtained from 7 animals in which the aortae had been perfuse-fixed with formaldehyde prior to wax embedding and 7 animals with glutaraldehyde for resin embedding. Cross-sectional areas of total aortic wall measured by MRI in 27 randomly selected images from 11 animals in study 2 and their corresponding intima plus media cross-sectional areas measured histologically did not correlate well, probably because many of the lesions had regressed and therefore

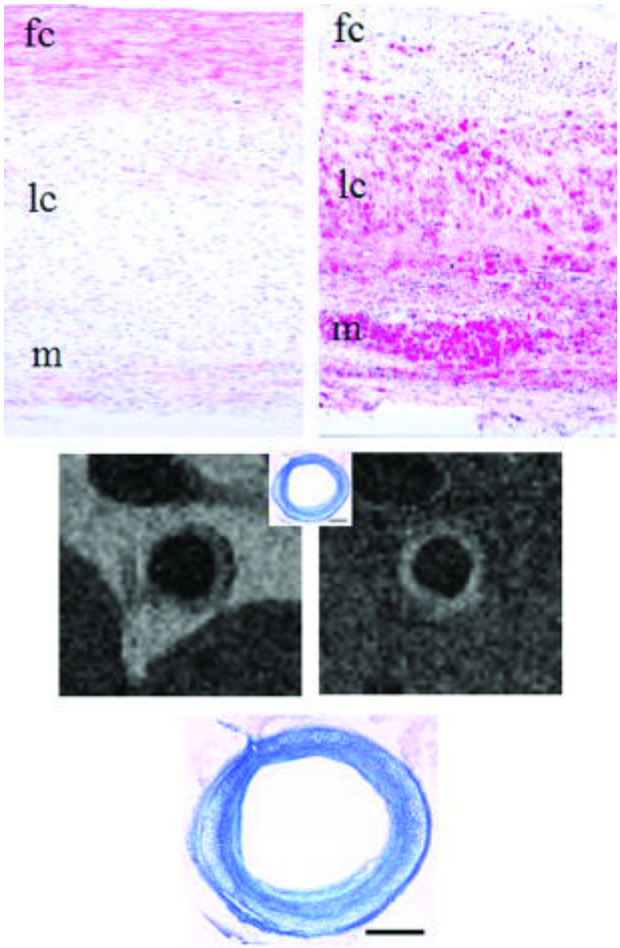

Figure 1. *The images (a) and (b) show immunohistochemical staining of an atherosclerotic lesion in a cholesterol fed NZW rabbit. Under the fibrous cap (fc), the cells of which were stained for SM-actin (left), a lipid core (lc) is found containing large amounts of extracellular lipid and lipid laden macrophages stained by RAM11 antibody (right) (m: media). Images c) and d) show NFS and FS MRI of a rabbit aorta in study 1. Histological section e), included also as a scaled image, shows the matched identical region of the same aorta following resin embedding (scale bar = 1 mm). The thickness of the lesion and its components are well within the resolution of MRI (250x250 µm in plane x 2000 µm thick).*

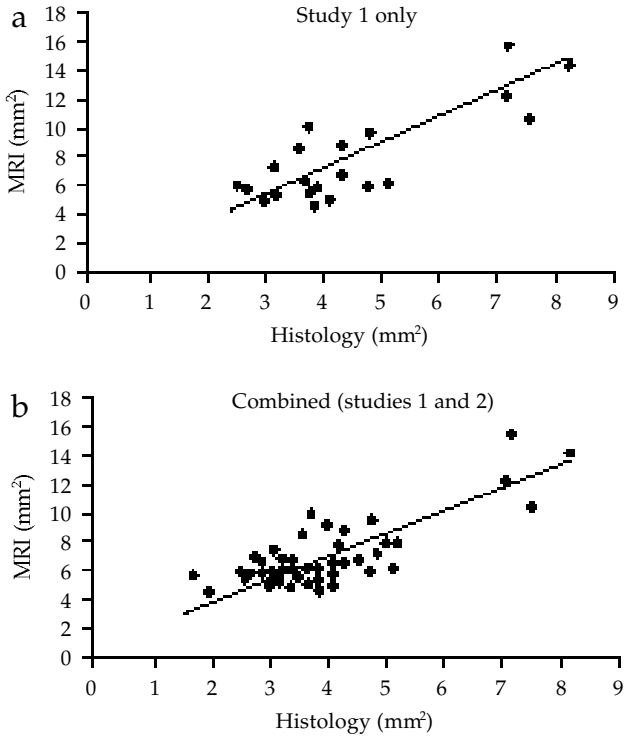

Figure 2. *Correlation between cross-sectional areas of total aortic wall measured by MRI and cross-sectional areas of aortic intima plus media measured by histology in animals from* ***(a)*** *study 1 and* ***(b)*** *study 1 plus study 2.*

the mean lesion size was quite small. The data from study 2 was then combined with those from study 1 to determine the correlation between MRI and histology. The results, therefore, include atherosclerotic lesions measured during progression and regression. We found a similar close correlation between MRI and histological data in this expanded set of lesions (r_{1+2} = 0.805, slope p_{1+2} <0.01) as was found in study 1 lesions alone (*Figure 2*).

Discussion

In the present study we describe a high-resolution MRI method used to image atherosclerotic lesions in the aortae of cholesterol-fed NZW rabbits. Ideally, we would have been able to measure changes in the volume of plaque in the aortic intima. However, as MRI could not distinguish the boundary between the intima and media we, therefore, used it to measure changes in the volume of the whole aortic wall and vessel lumen. This was considered a suitable surrogate measurement as changes in wall volume during atherogenesis are predominantly caused by changes in intimal volume.

In study 1, balloon injury of the aortae and 30 weeks of cholesterol feeding induced variable sized lesions which, if greater than 300 µm in thickness were visible by MRI. There were areas of loss of signal intensity within the aortic walls which coincided with distinct morphological differences including calcification, lipid accumulation and lack of lesion. However, these different morphologies could not be discriminated using the present MRI protocol. This observation is consistent with the results of Fayad et al and Helft et al who found that complex analysis of different relaxations and proton density is required to identify certain components of atherosclerotic plaques.[9,14] It is well known that lipid composition determines its MR intensity and this may cause the image intensity of fat in plaques of rabbits to differ from that of humans.[15] The present study confirms the work of Trouard et al that fat in rabbit plaques produces relatively hypointensive T2 weighted signals.[15]

In our first study 5 rabbits were imaged at a single time point and aortic wall cross-sectional areas measured using fat suppressed MRI. We showed that these data correlated well (r_1=0.83) with vessel wall cross-sectional areas mea-

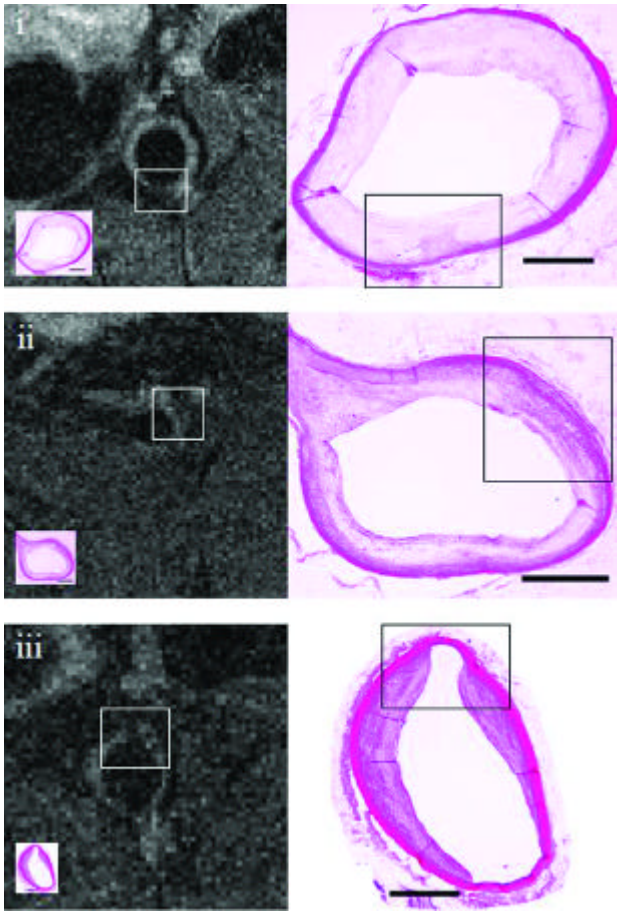

Figure 3. *Heterogeneities in MR images (fat-suppressed, T2 weighted) of rabbit aortae, in study 1, showing coincidence with distinct histological differences in their aortae. Hypointensity caused by calcification (i), region of lipid core (ii) and non-lesioned area (iii) (scale bar = 1 mm).*

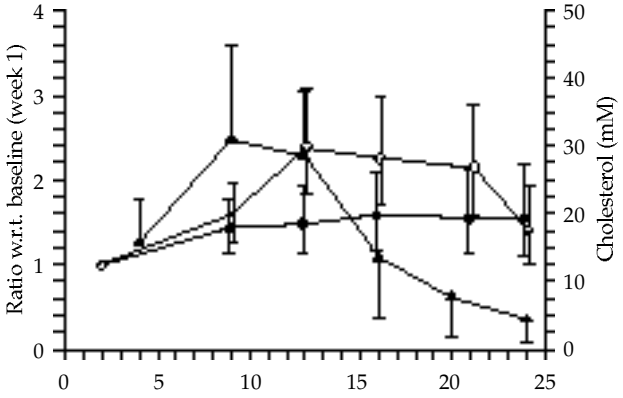

Figure 4. *Change in aortic wall (empty circle) and lumen volume (filled circle) during the course of study 2 expressed as mean ratio to baseline with 95% confidence intervals. (Data points have been displaced slightly for clarity.) Serum cholesterol levels (filled triangles) are also shown.*

sured histologically. In the subsequent study serial MRI was performed on a further 18 rabbits to examine the time course of lesion development and remodelling of the aorta. When MRI and histological data from both studies were combined the correlation coefficient (r_{1+2}) was only changed slightly to 0.805. Similarly good correlations between MRI and histology have been described previously in studies in genetically engineered mice,[7,16] rabbits[8,14] and humans.[9]

It has been shown that human atherosclerotic arteries are able to remodel by increasing their external circumference in order to accommodate plaques without significant reduction in lumen areas.[6] MRI has been used to show remodelling of atherosclerotic aortae in rabbits in two previous long term studies conducted over six months[10,17] and in genetically modified mice.[18] However, in the present study we were able to show significant progression of atherosclerotic lesions in the aortae of cholesterol-fed NZW rabbits within 3 months as well as regression in aortic wall volume after only another 3 months on a plasma cholesterol lowering regime as opposed to 6 and 8 months in the earlier studies. Lumen volume increased by week 8 then remained unchanged suggesting rapid remodelling of the atherosclerotic aortae. Arterial remodelling occurred during lesion progression and persisted through regression. The reasons for remodelling are not known although haemodynamic effects may partly explain it.[19]

We have shown that atherosclerotic lesions that develop within 12 weeks in rabbit aortae as a result of high-fat diet feeding and balloon injury can be visualised by MRI. There was also a good correlation between aortic lesion (wall) areas measured by MRI and histology. Using MRI we were able to show not only progression but also regression of lesions in our serial MR imaging study. In this experiment we demonstrated, for the fist time in a short-term study, remodelling of rabbit aortae. In conclusion, high-resolution MRI is a useful method to follow dynamic changes in experimental models of atherosclerosis, and may also prove beneficial in monitoring lesion development on treatment with potential anti-atherogenic agents.

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