Coronary Artery Disease

Local Arterial Stiffening Assessed by MRI Precedes Atherosclerotic Plaque Formation

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Background—Atherosclerosis is known to impair vascular function and cause vascular stiffening. The aim of this study was to evaluate the potential predictive role of vascular stiffening in the early detection of atherosclerosis. Therefore, we investigated the time course of early functional and morphological alterations of the vessel wall in a murine atherosclerosis model. Because initial lesions are distributed inhomogeneously in early-stage atherosclerosis, MR microscopy was performed to measure vascular elasticity locally, specifically the local pulse wave velocity and the arterial wall thickness.

Methods and Results—Local pulse wave velocity and the mean arterial wall thickness were determined in the ascending and the abdominal aortae of ApoE−/− and wild-type mice. In vivo MRI revealed that baseline pulse wave velocity and morphology were similar in 6-week-old ApoE−/− and WT mice, whereas at the age of 18 weeks, local pulse wave velocity was significantly elevated in ApoE−/− mice. Significantly increased vessel wall thickness was not found in ApoE−/− mice until the age of 30 weeks. Histological analysis of the aortae of ApoE−/− and WT mice showed that increased pulse wave velocity coincided with the fragmentation of the elastic laminae in the arterial wall, which is hypothesized to induce early vascular stiffening and may be promoted by macrophage-mediated matrix degradation.

Conclusions—We newly report that the assessment of local pulse wave velocity via MRI provides early information about the local progression of atherosclerosis before macroscopic alterations of the vessel wall occur. (Circ Cardiovasc Imaging. 2013;6:916-923.)

Key Words: arterial stiffness ■ atherosclerosis ■ elastin fragmentation ■ local pulse wave velocity ■ magnetic resonance imaging

To manage the increasing number of patients with cardiovascular disease in an aging population,1 strategies for therapeutic intervention in the early stages of the disease are needed to prevent adverse events such as myocardial infarction or stroke. Major components for accomplishing this goal are a detailed understanding of the pathophysiology of early-stage atherosclerosis and reliable techniques to detect those.

Clinical Perspective on p 923

Atherosclerosis is known to lead to decreased vascular elasticity and compliance because of increased arterial stiffness.2 Up to now most clinical applications evaluating vessel wall stiffness are focused on the measurement of parameters reflecting global vessel wall elasticity, such as the carotid-femoral-pulse wave velocity (PWV).3–7 The formation of initial atherosclerotic lesions, however, is a local process8 requiring imaging capabilities that account for the heterogeneous nature of plaque distribution. We hypothesized that, in particular, early alterations of local vascular stiffness provide a high predictive value for successive lesion development. However, in contrast to the determination of global PWV, which merely reflects the average travelling speed, noninvasive access of the local PWV is challenging. Therefore, we recently introduced an MRI method that allows the examination of local PWV.9,10 In the present study, we applied this technique to monitor the natural progression of atherosclerosis in the murine aorta and correlated local PWV with vessel wall thickness in serial measurements. Both parameters were examined in vivo in wild-type (WT) control mice and apolipoprotein E–deficient (ApoE−/−) mice, which spontaneously develop atherosclerotic lesions of morphology similar to those observed in humans.11,12 Local PWV and vessel wall thickness were evaluated by time-resolved flow and morphology measurement using ultrahigh-field MR microscopy at 17.6 T. In addition, we performed histological examinations to investigate structural changes of the vessel wall at the time of imaging.

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Methods

All in vivo MR experiments were performed on a 17.6-T MRI system (Bruker Avance 750, Bruker Biospin, Rhein-stetten, Germany) with an 89-mm vertical bore. As radio frequency transmission and receiver coils, we used 2 custom-built birdcage resonators with an inner diameter of 20 mm for mice of <24.0 g bodyweight and of 25 mm for mice >24.0 g bodyweight. For heartbeat synchronized sequence timing and the suppression of respiratory motion artifacts, a respiratory-gated cardiac trigger was realized by the use of a pneumatic sensing balloon and a custom-built trigger unit.

Local PWV and vessel wall thickness were measured in the ascending aorta, representing a predilection site for plaque formation, and in the upper abdominal aorta, representing a location with mild atherosclerotic burden (Figure 1). For the localization of the ascending and the abdominal aorta, a set of 2-dimensional fast-low-angle-shot (FLASH) experiments was performed. MR measurements in the ascending aorta were conducted at the level of the crossing of the pulmonary artery and in the abdominal aorta at a level of ~2 mm below the diaphragm.

Animal Protocol

A total of 80 mice were used in this study. Four-week-old female ApoE−/− and WT C57Bl/6 mice were obtained from Charles River Laboratories (Sulzfeld, Germany). The ApoE−/− mice (B6.129P2-ApoEtm1Unc/J, Stock Number: 002052) had been backcrossed into the C57BL/6J genetic background for at least 10 generations. The ApoE−/− groups were placed on an atherogenic Western-type diet (TD88137, ssniff GmbH, Soest, Germany) starting at the age of 4 weeks, whereas the WT control mice were fed a regular chow diet for the duration of the study. The Western-type diet contains more fat and cholesterol than a normal chow diet and accelerates the formation of atherosclerosis in ApoE−/− mice. WT C57Bl/6 mice do not develop significant atherosclerosis.

MR experiments with morphological examination and local PWV measurements were performed in 2 groups consisting of 14 WT C57Bl/6 and 18 ApoE−/− mice. Out of these groups, varying subsets of 10 WT C57Bl/6 and 12 ApoE−/− mice were imaged serially at the age of 6, 18, and 30 weeks in a follow-up setup (see online-only Data Supplement for detail). For histological examination of the elastin fragmentation, additional 24 ApoE−/− and 24 WT C57Bl/6 mice were euthanized. Eight mice from each group were euthanized at the age of 6, 18, and 30 weeks.

All experimental procedures were in accordance with institutional guidelines and were approved by the Institute of Animal Care of the district government of Lower Franconia.

Morphological MRI

For imaging vessel wall morphology, a multi-slice-multi-spin-echo (MSME) sequence was used (slices = 8; echoes = 3; averages = 3; TE=9 ms; TR=1000 ms; matrix = 256 × 256; pixel size = 78 × 78 × 400 μm3), which was positioned perpendicular to the aorta. To realize a black blood effect, a trigger delay was applied starting data acquisition at the beginning of systolic flow. The obtained images were analyzed using Amira 3.1 (Visage Imaging, San Diego, CA). For that, the luminal area and the whole cross-sectional area of the vessel were measured by computer-assisted planimetry (Figure 1H). Because the vessel wall area depends linearly on the vessel diameter and the vessel diameter was not equal for all mouse groups (Table), we report the mean vessel wall thickness. The mean vessel wall thickness was determined by subtracting the inner and outer vessel radii, which were calculated out of the luminal and whole cross-sectional vessel area. This was done in 3 subsequent slices in the abdominal aorta, respectively, in 2 to 3 subsequent slices in the ascending aorta depending on the length of the ascending aorta.

MRI of the Local PWV

Under the assumption of a reflectionless and unidirectional waveform in the early systolic flow pulse, the local PWV can be approximated by PWV=δQ/δA (Q(t), volume flow through the vessel; A(t), cross-sectional area of the vessel) with the data of a single slice.9,11 For the measurement of the time course of the parameters Q and A, a high-resolution phase-contrast (PC) Cine-FLASH sequence was performed perpendicular to the aorta with through plane flow encoding (slices, 1; TE=1.7 ms; TR=5 ms; matrix, 150 × 150; pixel size, 147 × 147 × 1000 μm3; frames per heart cycle, 40). By the use of an interleaved acquisition mode, a temporal resolution of 1 ms could be achieved. This method is sensitive to cardiac arrhythmia; consequently, datasets

Figure 1. In vivo determination of local pulse wave velocity (local PWV) (A–D) and arterial wall thickness (F–H) in the ascending and abdominal aorta. Slice positioning for the local PWV measurements (green) and arterial wall thickness measurements (white) is shown in a fast-low-angle-shot (FLASH) navigation scan (E). Local PWV is assessed by simultaneously recording cross-sectional area (A) and flow (D) through the aorta in early systole. The slope of the flow/area plot (C) represents the local PWV at the examined location (B, exemplarily shown for the ascending aorta; phase-contrast-Cine-FLASH magnitude image). Measurement of the vessel wall thickness is performed in 3 subsequent slices for each location (F–H) show representative multi-slice-multi-spin-echo (MSME) slices for the abdominal aorta; H, enlargement with segmentation details. The mean arterial wall thickness (ΔR) is determined by segmenting the lumen and the whole cross-sectional area of the vessel and calculating the inner radius (Ri) and outer (Ro) radius.
were considered not evaluable in cases of fluctuation of the duration of the heart cycle of 3 ms or more over the duration of a local PWV measurement. The evaluation was performed with the help of Matlab (The MathWorks, Natick, MA) and Amira 3.1 (Visage Imaging, San Diego, CA) software. See online-only Data Supplement for detailed information about the local PWV MR sequence and data analysis.

Histological Analysis

The aortae were excised and perfused with Tissue Tek O.C.T. Compound (Sakura Finetek Europe B.V., Alphen aan den Rijn, The Netherlands), fixed in liquid nitrogen, and stored at −80°C. Serial, transversely cut 8 µm sections of the upper abdominal aorta were collected. For visualization of the elastic laminae, the sections were stained with Weigert’s resorcine fuchsin component of the Elastica van Gieson staining kit (Merck KGaA, Darmstadt, Germany). Elastin fragmentation was quantified by counting the number of fractures in the elastic laminae in 3 subsequent sections of the upper abdominal aorta. Simultaneously, the aortic media cross-sectional area was traced manually. The density of elastin fragmentation was expressed as a percentage of the aortic media cross-sectional area.

In all groups, immunohistochemical staining for macrophages (CD68; AbD Serotec, Oxford, UK) was performed. Immunopositive areas were segmented by computerized planimetry using ImageJ (National Institute of Health) and expressed as a percentage of the total vessel wall area. In the 18-week-old groups, additional double immunofluorescence staining for CD68 together with matrix metalloproteinase-9 (MMP-9; Aviva Systems Biology, San Diego, CA) and for CD68 together with neutrophil elastase (NE; Abcam, Cambridge, UK) was performed. Sections were counterstained with 4',6-diamidino-2-phenylindole for visualization of nuclei.

Statistical Analysis

All data are expressed as mean±SEM. Statistical analysis was conducted with SPSS software (SPSS, Chicago, IL). Normal distribution of data was confirmed using the Shapiro–Wilk test, and homogeneity of variances was tested with Levene’s test. Differences between ApoE−/− and WT control mice were tested using an unpaired 2-tailed Student t test for normally distributed data and a nonparametric Mann–Whitney U test for not normally distributed data. Changes over time in the individual groups were evaluated using a linear mixed effects model with Bonferroni post hoc comparison. A P value of <0.05 was considered statistically significant, and a P value of <0.01 was considered highly significant.

Results

In Vivo MRI of the Vessel Wall Morphology

In the ascending aortae of ApoE−/− mice, a slight thickening of the vessel wall could be observed at the age of 18 weeks. The mean vessel wall thickness increased significantly (P<0.003) compared with 6-week-old ApoE−/− mice. However, because of a small growth of vessel wall thickness in the WT control group between the age of 6 and 18 weeks, no significant differences were observed in these groups at the age of 18 weeks (6 weeks: ApoE−/− 96±2 versus WT 96±1 µm, P=0.901; 18 weeks: ApoE−/− 108±4 versus WT 100±2 µm, P=0.093). At the age of 30 weeks, ApoE−/− mice showed massive accumulation of atherosclerotic plaque in the ascending aortae, which results in a highly significant increase in mean vessel wall thickness compared with the WT group (30 weeks: ApoE−/− 142±5 versus WT 103±3 µm, P<0.001; Figures 2A and 3A). Morphological MRI of the abdominal aorta revealed no significant difference in vessel wall thickness between ApoE−/− and WT control mice at the age of 6 and 18 weeks (6 weeks: ApoE−/− 93±3 versus WT 91±4 µm, P=0.54; 18 weeks: ApoE−/− 95±3 versus WT 93±1 µm, P=0.56). At the age of 30 weeks, first formation of atherosclerotic plaque became visible in the abdominal aortae of ApoE−/− mice, and mean vessel wall thickness increased

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**Table. Weights and Arterial Diameters of the Different Groups**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Weight, g</th>
<th>Ascending Aorta</th>
<th>Abdominal Aorta</th>
</tr>
</thead>
<tbody>
<tr>
<td>ApoE−/−</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 wk</td>
<td>19.2±0.4*</td>
<td>1.39±0.04</td>
<td>1.08±0.02</td>
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<tr>
<td>n=10</td>
<td>n=11</td>
<td>n=12</td>
<td></td>
</tr>
<tr>
<td>18 wk</td>
<td>25.1±0.6</td>
<td>1.61±0.04</td>
<td>1.18±0.03</td>
</tr>
<tr>
<td>n=12</td>
<td>n=9</td>
<td>n=11</td>
<td></td>
</tr>
<tr>
<td>30 wk</td>
<td>28.5±0.8</td>
<td>1.68±0.03†</td>
<td>1.22±0.03†</td>
</tr>
<tr>
<td>n=12</td>
<td>n=9</td>
<td>n=9</td>
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</tr>
<tr>
<td>WT control</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>6 wk</td>
<td>18.1±0.3</td>
<td>1.38±0.01</td>
<td>1.03±0.02</td>
</tr>
<tr>
<td>n=10</td>
<td>n=10</td>
<td>n=10</td>
<td></td>
</tr>
<tr>
<td>18 wk</td>
<td>26.0±0.8</td>
<td>1.52±0.03</td>
<td>1.11±0.03</td>
</tr>
<tr>
<td>n=10</td>
<td>n=10</td>
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<td></td>
</tr>
<tr>
<td>30 wk</td>
<td>31.5±1.4</td>
<td>1.56±0.04</td>
<td>1.11±0.03</td>
</tr>
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<td>n=10</td>
<td>n=8</td>
<td>n=9</td>
<td></td>
</tr>
</tbody>
</table>

Data are presented as mean±SEM. †P<0.05; *P<0.01 vs WT control.
highly significantly compared with 18-week-old ApoE−/− mice and age-matched WT control mice (30 weeks: ApoE−/− 117±3 versus WT 99±4 µm, \(P<0.01\); Figures 2B and 3C).

**Local Vascular Stiffening Detected by MRI Precedes Morphological Alterations**

Baseline local PWV in the ascending aorta was similar in ApoE−/− and WT control mice at the age of 6 weeks (6 weeks: ApoE−/− 1.7±0.1 versus WT 1.8±0.2 m/s, \(P=0.911\)). Between the 6th and the 18th week, local PWV increased highly significantly in the ApoE−/− group and was significantly elevated compared with the level of the age-matched control group at the age of 18 and 30 weeks (18 weeks: ApoE−/− 2.6±0.1 versus WT 1.9±0.2 m/s, \(P<0.01\); 30 weeks: ApoE−/− 2.9±0.3 versus WT 2.0±0.1 m/s, \(P=0.018\); Figure 3B).

The elastic properties of the abdominal aorta characterized by the local PWV showed a similar dynamic like the elastic properties of the ascending aorta. In the abdominal aorta, local PWV was significantly elevated in ApoE−/− mice compared with WT mice at the age of 18 and 30 weeks (18 weeks: ApoE−/− 2.6±0.1 versus WT 1.9±0.2 m/s, \(P<0.01\); 30 weeks: ApoE−/− 2.9±0.3 versus WT 2.0±0.1 m/s, \(P=0.018\); Figure 3B).

**Elastin Fragmentation and Macrophage Infiltration Coincide With Elevated PWV Values**

At 18 weeks, the abdominal aortae of ApoE−/− mice exhibited increased local PWV without simultaneous occurrence of macroscopic alterations of the vessel wall. To investigate possible changes in the ultrastructural composition of the vessel wall, we performed histological analyses of the abdominal aorta in age-matched ApoE−/− and WT control mice. We determined elastin fragmentation, which has been proposed to contribute to arterial stiffening.14 The elastin fragmentation was quantified by counting the number of interruptions in elastic laminae and normalizing it to the aortic media cross-sectional area. The density of elastin fractures was not significantly different between both groups at the age of 6 weeks (\(P=0.23\)). In older ApoE−/− mice, the elastin fragmentation increased significantly compared with WT mice by 47% (\(P=0.011\)) at the age of 18 weeks and by 50% (\(P=0.013\)) at the age of 30 weeks (Figure 4). To investigate the cause of elastin fragmentation, we analyzed the inflammatory activity in the vessel wall over time. By fluorescence microscopy, macrophage infiltration was significantly elevated in the vessel walls of 18-week-old ApoE−/− mice (\(P<0.01\)) and 30-week-old ApoE−/− mice (\(P<0.001\)). Double immunofluorescence revealed that macrophages were colocalized with the elastolytic enzymes MMP-9 and neutrophil elastase (Figure 5A and 5B).

**Mice Weights and Arterial Diameters**

There was no significant difference in mean weight between ApoE−/− and WT control mice except at the age of 6 weeks, where ApoE−/− mice showed a slightly but significantly elevated weight (ApoE−/− 19.2±0.4 versus WT 18.1±0.3 g,
The arterial diameters were similar between ApoE−/− and WT mice at the age of 6 and 18 weeks; however, at the age of 30 weeks, ApoE−/− mice exhibited moderate but significant dilatation of the ascending and the abdominal aorta (ascending aorta: ApoE−/− 1.68±0.03 versus WT 1.56±0.04 mm, \(P=0.043\); abdominal aorta: ApoE−/− 1.22±0.03 versus WT 1.11±0.03 mm, \(P=0.013\)). A synopsis of all data is presented in the Table.

Discussion

MRI has emerged as the leading imaging modality to characterize and monitor advanced atherosclerotic lesions in vivo regarding their vulnerability, inflammatory activity, or impairment of endothelial function.\(^{15-20}\) Providing reliable techniques to identify atherosclerotic lesions in the early stages, however, is still challenging. In this study, we could show that alterations of local vascular elasticity precede the formation of atherosclerotic lesions.

We used in vivo MR microscopy to investigate the time course of the vessel wall thickness and local PWV during early stages of atherosclerosis in ApoE−/− and WT control mice. Although initial PWV and vessel wall thickness were similar between the 2 groups at the age of 6 weeks, ApoE−/− mice exhibited a significant increase of the local PWV at the age of 18 weeks. Morphological manifestations of atherosclerosis reflected in significant vessel wall thickening, however, could not be detected until the age of 30 weeks.

In previous studies, MRI has proven its capability to measure the size of atherosclerotic lesions accurately at

Figure 4. In histopathologic sections of the abdominal aorta, the elastic laminae are visualized by Weigert’s resorcin fuchsin staining (A–C). The lower row shows enlargements of characteristic sections of the vessel wall. Only few elastin fractures are seen in 6-week-old ApoE−/− mice (A). 18-week-old ApoE−/− mice exhibit more than twice as many elastin fractures (black arrows, B). In 30-week-old ApoE−/− mice, elastin fractures are often found beneath atherosclerotic plaques (black arrow, C). The density of elastin fractures is significantly increased in ApoE−/− mice compared with wild-type (WT) mice at the age of 18 and 30 weeks (D) (*\(P<0.02\), scale bar: 200 \(\mu\)m).

Figure 5. Macrophage infiltration to the vessel wall of the abdominal aorta increases significantly in 18-week-old ApoE−/− mice and is colocalized with matrix metalloproteinase-9 (MMP-9) and neutrophil elastase expression. A, The subintimal infiltration of macrophages (left). Fractured elastic laminae (white arrows) are contoured white (derived from elastin autofluorescence). Neutrophil elastase is strongly colocalized with CD68-positive areas, and MMP-9 is also colocalized with CD68 but occurs in deeper layers too. The merged images with additional 4′,6-diamidino-2-phenylindole staining are shown on the right (scale bar: 20 \(\mu\)m). The CD68-positive area is highly significantly increased in 18- and 30-week-old ApoE−/− mice (B, *\(P<0.01\), **\(P<0.001\)). WT indicates wild type.
The results of our morphological measurements are in close agreement with a previous study by Choudhury et al., who performed serial morphological MRI measurements of the development of atherosclerosis in the abdominal aortae of ApoE−/− mice and demonstrated that the aortae remained free of atherosclerosis until the age of 20 weeks. Those studies, however, did not investigate the elastic properties of the vessel wall.

Although MRI has recently been used to quantify impaired vascular elasticity via global PWV, vascular distensibility, and endothelial function in humans, those studies were limited to patients with morphologically manifest atherosclerosis. PWV and vascular distensibility are parameters describing the arterial stiffness, which is primarily determined by the extracellular matrix, whereas the endothelial function measures the ability of the endothelium to regulate vascular tone in response to a variety of stimuli such as acetylcholine or physical exercise. Lately, it was shown that contrast-enhanced MRI using iron oxide- and gadolinium-based contrast agents can visualize atherosclerotic lesions in young ApoE−/− mice in the brachiocephalic artery, a predilection site for atherosclerotic lesions. A recent study from our group indicated that the change in cross-sectional vessel area during systole, a parameter related to vascular elasticity, is already impaired in the early stages of atherosclerosis. The systolic cross-sectional change in vessel area, however, is not an independent parameter of vascular elasticity because it depends on cardiac output. In the presented study, therefore, we assessed the local PWV, which is quantitatively related to the vessel wall elasticity by the Moens–Korteweg equation. A major novelty of this study is the observation of abnormal vascular stiffness before atherosclerotic lesions are detected. This finding suggests that the local PWV provides superior sensitivity for detecting local variations of vascular elasticity, which may be masked by the commonly used global PWV. An important confounder for determining vascular stiffness is blood pressure. Because of the nonlinear elastic properties of the vessel wall, increased distending pressure leads to decreased distensibility and results, therefore, in elevated PWV values. However, ApoE−/− mice are known to be normotensive in the early and medium stages of atherosclerosis.

The impairment of vascular function in ApoE−/− mice without accompanying observable morphological manifestation of atherosclerosis implies possible ultrastructural alterations of the vessel wall. Morphological investigations imply that arterial stiffening may be caused by fragmentation of elastin laminae in the vessel wall. Hence, we quantified elastin fragmentation by determining the number of fractures in the elastic laminae and normalizing it to the cross-sectional area of the aortic media. Thereby, a quantitative parameter describing the density of elastin fractures was created. We found that elastin fragmentation in ApoE−/− mice rises significantly compared with WT mice before a thickening of the vessel wall could be observed by in vivo MRI. Overall elastin fragmentation and local PWV showed similar temporal dynamics. These findings support the concept of elastin fragmentation playing a major role in early vascular stiffening. O’Rourke introduced a model explaining the causal chain between elastin fragmentation and vascular stiffening. According to this model, the elastic laminae are parallel to the stiffer collagenous fibers in the vessel wall. Thus, by fragmentation of the elastic laminae, stress is consequently transferred to the more rigid collagenous components of the arterial wall, thereby resulting in vascular stiffening. The initial cause of elastin fragmentation, however, is still a subject of ongoing research. Enzymatic degradation accompanying inflammatory processes is likely to play a key role. Studies in human and murine atherosclerosis have shown the crucial role of macrophage-induced expression of elastolytic active MMP-9 and neutrophil elastase for matrix breakdown and plaque rupture of advanced atherosclerotic lesions. A previous observational study in patients with isolated systolic hypertension showed a positive correlation between serum MMP-9 levels and arterial stiffness, suggesting that MMP-9 may be involved in the process of arterial stiffening. Using fluorescence microscopy, we found that vascular stiffening and elastin fragmentation were accompanied by macrophage infiltration in the vessel wall. Macrophage infiltration was colocated with MMP-9 and neutrophil elastase expression in ApoE−/− mice at an age when vascular stiffening was present. This implies that the novel approach of local PWV measurements allows for visualizing and monitoring the effect of initial inflammatory processes in the vessel wall before morphological alterations can be determined. In addition to matrix breakdown, macrophage infiltration is also known to trigger ineffective elastogenesis, which potentially modulates vascular elasticity. In future studies, the new approach of noninvasively assessing atherosclerotic plaque burden and plaque elastin content by the use of an elastin-specific MR contrast agent and the local PWV determination may provide synergistic information to generate a deeper understanding of the significance of elastin breakdown and ineffective elastogenesis in early-stage atherosclerosis.

In human atherosclerosis, the onset and temporal development of local vascular stiffening is unknown. A recent study in hypercholesterolemic children reported that global PWV, but not intima-media thickness, is an early indicator of vascular damage in this particular patient group. This report emphasizes the relevance of local vascular stiffening in human atherosclerosis and stresses the need for further investigation of the interrelation between atherosclerosis and the elastic properties of the vessel in the early stages of the disease. Because the local PWV measurements can be done using clinically available MR hardware and no approval for contrast agents or other reagents is needed, translation of this method to patients with cardiovascular disease is feasible to further investigate the significance of early arterial stiffening in the development of human atherosclerosis. Thus, future clinical application of this method may allow for early detection of atherosclerosis and might thereby provide a window of opportunity for diagnosis and therapeutic intervention well before symptoms occur. In an experimental setup, the technique has potential to advance basic knowledge of the role of vascular stiffening in the pathophysiology of atherosclerosis and other conditions such as Marfan syndrome and aortic aneurysms.
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Disclosures
None.

References
CLINICAL PERSPECTIVE

Despite advances in cardiovascular care, atherosclerosis and accompanied cardiovascular disease are still one of the major causes for mortality and morbidity in the Western world. Adverse events such as myocardial infarction or stroke occur in the advanced stages of the disease, whereas subclinical stages often exist decades before. Thus, reliable techniques to detect early-stage atherosclerosis could enable therapeutic interventions to prevent future morbidity. Parameters of arterial stiffness such as the pulse wave velocity (PWV) are known to be increased in atherosclerotic vessels. Although the formation of initial atherosclerotic lesions is a local process, most applications measure the arterial stiffness globally, for example, the carotid-femoral PWV. In this study, we therefore investigated the local temporal evolution of vascular stiffening and vessel wall thickening in the ApoE−/− mouse model. Using ultrahigh-field MRI at 17.6 T, we found that a significant increase in local PWV occurred before significant morphological alterations of the vessel wall could be observed. Our histological investigations imply that fragmentation of the elastic laminae in the arterial wall, which may be promoted by macrophage-mediated matrix degradation, possibly contributes to this effect. Potential clinical implications of these findings should be explored in a prospective clinical study to investigate the predictive value of early arterial stiffening and to provide new insights on the pathophysiology of early atherosclerotic lesions.
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SUPPLEMENTAL MATERIAL

Supplemental methods

*Magnetic resonance imaging of the local PWV*

The Q/A-method assumes that the pulse wave is free of reflections so that the pulse wave velocity can be estimated as a function of the blood volume flow Q(t) and the cross-sectional area A(t). Using the expression for the characteristic impedance and the wave equation for inviscid flow in an elastic tube, it can be shown that

\[ \text{PWV} = \frac{dQ}{dA}. \]

Therefore, the local pulse wave velocity can be estimated by measuring the time course of the blood volume flow Q and the cross-sectional area A during the early systolic pulse wave.

The basic imaging sequence applied to study the local PWV consisted of a 2D fast low angle shot (FLASH) sequence with velocity compensated gradients for all three gradient directions. Through-plane motion encoding was performed using bipolar gradients, directly added on the shape of the motion-compensating gradients as shown in Suppl. Fig. 1. Overall, three motion encoding data sets were acquired (v_{enc}=1.7 m/s).

To improve the native temporal resolution restricted by the repetition time (TR=5 ms), five interleaved time-delayed imaging experiments were performed, starting with variable delays of \( \Delta t = \{0, 1, 2, 3, 4\} \) ms as depicted in Suppl. Fig. 1. Thus, the dataset allows for a temporal data-sampling of one frame per millisecond during early systole. The duration of one local PWV measurement was approx. 6 minutes. For the duration of one local PWV measurement, the heart rate needed to be constant. Datasets with fluctuation of the duration of the heart cycle of 3 ms or more during the measurement were considered not evaluable. Out of a total of 132 local PWV measurements, 16 datasets had to be excluded due to irregular heart rate.
Velocity data were computed pixel-wise by fitting a line to the phase data as a function of the first moments of the bipolar encoding gradients. Suppl. Fig. 2 a/b shows the magnitude data with a corresponding phase-difference image (phase-data of a motion encoded image subtracted by the phase-data of a motion compensated image) of an early systolic time frame. A representative through-plane velocity profile is shown in Suppl. Fig. 2c.

The cross-sectional area of the vessel lumen was segmented manually for each time frame based on the magnitude images of the complex PC-Cine-FLASH datasets. Segmentations were repeated four times and the mean area was calculated for further computations. Blood volume flow was computed by adding pixelwise the volume flow of all intraluminal image pixels. Random high frequency changes of the time course of the volume flow- and cross-sectional areas have been reduced by applying a low-pass filter to the data. Subsequently, the onset of the systolic flow pulse was identified as the point of intersection of the linear extrapolation of the early systolic slope and the flow baseline at the end diastole. PWV was then determined as the slope of the linear QA-relation for the first five systolic data points. In phantom measurements, the local PWV values obtained using this MR based Q/A-method showed an excellent agreement with PWV values that were obtained using an intraluminal pressure sensor.

**Animal protocol**

For the MRI experiments, anesthesia was induced with 4% isoflurane and maintained with 1.5 - 2.0 vol.% isoflurane in 2 L/min pure oxygen during the MRI experiments. To exclude confounding effects on vascular function, first MRI measurements were performed after a minimum of 30 minutes of narcosis while the gradient cooling unit was used to maintain body temperature at 37°C. Suppl. Table 1 shows an exemplary timetable of an experiment.

For MR imaging, 18 ApoE<sup>−/−</sup> and 14 WT mice were obtained from Charles River Laboratories. Of those, 12 ApoE<sup>−/−</sup> and 10 WT mice were imaged at the age of 6, 18 and 30 weeks. Mice
were imaged serially, however, mice that had died or had to be excluded were replaced by littermates that had not been imaged before. Over the duration of the study, two ApoE<sup>−/−</sup> mice and one WT mouse died. Four ApoE<sup>−/−</sup> mice had to be excluded because they responded with respiratory impairment to the induction of anesthesia and one WT mouse had to be excluded for exceeding the bodyweight limit of the transmission and receiver coil.
<table>
<thead>
<tr>
<th>Procedure</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1  Anesthesia and positioning of the mouse in the scanner</td>
<td>20 min</td>
</tr>
<tr>
<td>2  Initialization and adjustment of the scanner</td>
<td>10 min</td>
</tr>
<tr>
<td>3  Set of FLASH navigation scans</td>
<td>10 min</td>
</tr>
<tr>
<td>4  Preparing morphological imaging of the ascending aorta, test scan</td>
<td>10 min</td>
</tr>
<tr>
<td>5  Morphological imaging of the ascending aorta (MSME)</td>
<td>20 min</td>
</tr>
<tr>
<td>6  Preparing local PWV measurement in the ascending aorta, test scan</td>
<td>4 min</td>
</tr>
<tr>
<td>7  Local PWV measurement in the ascending aorta (PC-Cine-FLASH)</td>
<td>6 min</td>
</tr>
<tr>
<td>8  Preparing morphological imaging of the abdominal aorta, test scan</td>
<td>10 min</td>
</tr>
<tr>
<td>9  Morphological imaging of the abdominal aorta (MSME)</td>
<td>20 min</td>
</tr>
<tr>
<td>10 Preparing local PWV measurement in the abdominal aorta, test scan</td>
<td>4 min</td>
</tr>
<tr>
<td>11 Local PWV measurement in the abdominal aorta (PC-Cine-FLASH)</td>
<td>6 min</td>
</tr>
<tr>
<td>12 Repeating failed scans if necessary</td>
<td>~ 20 min</td>
</tr>
<tr>
<td>13 Recovery of the animal</td>
<td>~ 10 min</td>
</tr>
</tbody>
</table>

**Total:** 120 – 150 min
Supplemental Figure 1. Pulse sequence schema and sequence timing schema of the PC-Cine-FLASH sequence

\[ \Delta t = (0 \text{ms}, 1 \text{ms}, 2 \text{ms}, 3 \text{ms}, 4 \text{ms}) \]; HF: radio frequency transmission; \( G_R \): frequency encoding gradient; \( G_P \): phase encoding gradient; \( G_S \): slice encoding gradient; \( \text{FE}_{QA} \): through-plane-flow-encoding

\[ \text{cine loop: } \ 8 \times 5 \text{ ms} \]
\[ \text{segment loop: } \ 5 \times (\Delta t+40 \text{ ms}) \]
\[ \text{phase encoding loop: } \times 150 \]
\[ \text{flow encoding loop: } \times 3 \]
Exemplary images of a PC-Cine-FLASH sequence for local PWV determination in the abdominal aorta. (a) Transversal PC-Cine-FLASH magnitude image of the upper abdominal aorta. The corresponding PC-Cine-FLASH phase-difference image (b) with through-plane motion encoding is used to derive the flow profile (c) of each time frame during early systole.
Supplemental References