Loss of Continuity in the Thin Epicardial Layer Because of Endomysial Fibrosis Increases the Complexity of Atrial Fibrillatory Conduction

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Background—The transition from persistent to permanent atrial fibrillation (AF) is associated with increased complexity of fibrillatory conduction. We have investigated the spatial distribution of fibrillation waves and structural alterations in the atrial free walls in a goat model of AF.

Methods and Results—AF was maintained for 3 weeks (short term [ST], persistent AF) or 6 months (long term [LT], permanent AF). Fibrillation patterns were assessed with epicardial mapping. The origin of fibrillation waves and sites of conduction abnormalities were more homogeneously distributed in LT than in ST goats. Histologically, the total area fraction occupied by fibrous tissue and the degree of perimysial fibrosis (separation between myocyte bundles) were not significantly different between groups. However, endomysial fibrosis (distance between myocytes within bundles) was significantly larger in LT goats, particularly in the outer millimeter of the atria. By contrast, myocyte diameters were larger in LT goats throughout the atrial walls. High-resolution optical mapping showed that epicardial wavefront expansion was slower and more anisotropic in LT than in ST goats. Finally, a mathematical model of a simplified atrial architecture confirmed the potential impact of epicardial endomysial fibrosis on AF complexity.

Conclusions—Altered propagation after 6 months of AF is consistent with homogeneous structural remodeling in the outer millimeter of the atria. Loss of continuity of the epicardial layer because of endomysial fibrosis may reduce its synchronizing effect, thereby increasing the complexity of fibrillatory conduction pathways. The exact distribution of fibrosis may be more important for the occurrence of conduction disturbances than the overall quantity. (Circ Arrhythm Electrophysiol. 2013;6:202-211.)

Key Words: atrial fibrillation ■ conduction ■ fibrosis ■ structure ■ tissue

In many patients suffering from atrial fibrillation (AF), the arrhythmia becomes more entrenched over months to years. This progression in AF stability is mimicked in several animal models. In goats with artificially maintained AF, AF episode duration gradually increases. Even after AF has become sustained (ie, not self-terminating) in this model, AF stability still gradually increases over the ensuing months. Sinus rhythm can be restored by pharmacological cardioversion after 3 to 4 weeks of AF (persistent AF), but not after 4 to 6 months of AF (permanent AF). We have previously demonstrated that the complexity of fibrillation patterns in the atrial free walls is higher in permanent AF than in persistent AF. This increased complexity, characterized by a higher number of smaller wavefronts, is thought to contribute to higher AF stability. A higher complexity of fibrillation patterns in the atrial free walls has also been observed in patients with chronic AF compared with patients with acute or paroxysmal AF. In paroxysmal AF patients, high-frequency activity in specific atrial areas, such as the pulmonary veins, may contribute disproportionately by triggering AF episodes. However, as atrial remodeling progresses and AF becomes more stable as a consequence, the entire atrial myocardium can contribute to the substrate for AF.

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A major candidate mechanism for causing the changes in fibrillatory conduction and the concomitant increase in AF stability is structural remodeling, which develops over the same slow time course of months. In the goat model of AF, structural remodeling includes fibrosis and myocyte hypertrophy, both of which can affect atrial conduction. The type (ie, reactive versus replacement) and exact distribution of fibrous tissue over the atrial walls are pertinent to its effect on atrial conduction, but have not been studied extensively. The atria have a complex anatomy, with a thin epicardial layer overlying an extensive endocardial...
We have recently reported that during the progression from acute to persistent and permanent AF, the degree of dissociation in fibrillation waves between the epicardium and the endocardial bundle network increases. Goats with acutely induced AF showed 18% dissociation of electrical activity between epicardium and endocardium, whereas dissociation had increased to 40% after 3 weeks of AF and to 70% after 6 months of AF. As a result, the atria become a more 3-dimensional substrate for AF, which is also reflected by an increased incidence of epicardial breakthrough (EB) waves, both in this goat model and in AF patients.

To elucidate the structural substrate for AF, we have analyzed the spatial distribution of fibrillation waves and structural changes in goats with 3 weeks (short term, ST) and 6 months (long term, LT) AF. We report that myocyte hypertrophy occurs throughout the atrial wall in LT, but that endomysial fibrosis occurs predominantly in the outer millimeter of the epicardium. Atrial epicardial wavefront propagation showed ubiquitous alterations throughout the free walls that are compatible with transverse uncoupling of myocyte strands because of endomysial fibrosis. Although the total amount of fibrosis is much smaller than in some other animal models of AF, we propose that the impact of this particular type of fibrosis on atrial conduction may be considerable, thus providing an interesting target for specific treatment strategies.

**Methods**

**Animal Model**
AF was maintained by burst pacing for 3 weeks (ST, n=10) or 6 months (LT, n=7). In open-chest follow-up experiments, propagation patterns in the atrial free walls were mapped during AF, and the spatial distribution of peripheral waves and EBs (waves appearing at the periphery and within the central part of the recording area, respectively) and slow conduction were analyzed (see the online-only Data Supplement).

**Histological Analysis**
After the electrophysiological measurements, complete atria of 5 ST and 5 LT goats were sectioned and stained with toluidine-blue to distinguish myocytes (dark blue) from connective tissue (light blue; see the online-only Data Supplement).

**Optical Mapping**
Optical mapping was performed in perfused hearts from separate groups of ST (n=8) and LT (n=6) goats. A 100×100 pixel camera was used to image the fluorescence of the voltage-sensitive dye.
di-4-ANEPPS (amino-naphthyl-ethenyl-pyridinium-propyl-sulfonate) in 2×2 cm areas of the left atrium (LA) free wall during stimulation within the field of view (see the online-only Data Supplement).

Mathematical Model
To investigate the effect of reduced transverse electrical coupling in the epicardial layer on AF complexity and the incidence of EBs, a dual-layer mathematical model was used (see the online-only Data Supplement).

Statistical Analysis
Histological data were analyzed using a linear mixed model (SAS v9.2, SAS Institute). Myocyte diameters were normally distributed, but intermyocyte and interbundle distances were log-transformed to correct right-skewness. Morisita indices, which were not normally distributed, were tested with a Kruskal–Wallis test and a Dunn’s post hoc test. Time series of wavefront spread were analyzed using a general linear regression model for repeated measures (SPSS v20, IBM Inc). A P<0.05 was considered statistically significant.

Results
Spatial Characteristics of Fibrillation Patterns
We have previously reported that in both atria of LT goats, compared with ST goats, conduction block is more frequent and the number of peripheral waves and EBs is larger, leading to a more complex, dissociated fibrillation pattern, whereas the AF cycle length or atrial fibrillation cycle length (AFCL) is not significantly different.4 Here, we have studied the spatial distribution pattern of fibrillation waves in the same goats. Figure 1A depicts the spatial pattern of EB waves. Compared with ST goats, the total number of EBs was higher in LT goats, but the spatial pattern was more homogeneous. The Morisita index, which reflects the degree of clustering, tended to be lower in the LT right atrium (RA) and was significantly lower in the LT LA, compared with ST atria. As apparent in Figure 1A, EBs tended to occur more frequently at some electrode sites than at others, particularly in ST goats. However, the average interval between EBs at a certain site was much longer than the AFCL in both atria of both groups (ST LA: 1334±444 ms, ST RA: 1328±133 ms, LT LA: 1199±130 ms; and LT RA: 891±90 ms). Thus, sites where EB originated did not behave as localized high-frequency sources.

Similarly, the number of peripheral waves was higher in LT goats, but distributed more homogeneously around the periphery (Figure 1B), leading to a lower Morisita index (significant for the LA). The incidence of long conduction times (>8 ms) between neighboring electrodes was also higher in LT atria, but again with a more dispersed pattern, leading to a lower Morisita index (significant for the LA). A similar analysis is shown in the online-only Data Supplement for the distribution of AFCLs (Figure S1A). There, however, the pattern in LT goats showed more clustering, translating into a higher Morisita index for both atria, because of regions within the recorded area with a lower activation frequency. The average AFCL was not systematically higher in either atrium: the RA had a shorter AFCL than the LA in 5 out of 10 ST goats and in 5 out of 7 LT goats (see Figure S1B).

Relevant Aspects of the Atrial Anatomy
The atrial walls have a complex anatomy (endocardial aspect in Figure 2A). The anterior parts possess an extensive network of trabeculae underlying the epicardial layer, which is as thin as 1 mm in many places (Figure 2B). Figure 2C and animated stacks of aligned atrial slices (see the online-only Data Supplement) illustrate that endocardial bundles give off branches to the epicardial layer at various sites along their length. These branches to the epicardium are often orientated perpendicularly to the underlying trabeculae. In this sense, the thin epicardial layer is formed by the final branches of the underlying trabecular network. Within small areas of the free wall such as in Figure 2C, a variety of epicardial fiber orientations was visible.

Distribution of Structural Remodeling
Because of LT AF
To help understand the difference in fibrillation pattern between ST and LT goats, we have studied the distribution of fibrous tissue and myocyte size. No large areas of scar tissue were apparent in any of the atria investigated. Moreover, the overall amount of intramyocardial fibrous tissue was not different between ST and LT goats in either atrium (see Figure S2). In Figure 3A, we have quantified the number of larger interruptions (>0.5 mm) in the epicardial layer. The number of interruptions was higher in the RA than in the LA, but did not differ between the ST and LT groups in either atrium.

Figure 2. Anatomic structure of the atria. A, Excised and back-illuminated atrial preparation shows the extensive trabecular network underlying the thin epicardial layer. CT indicates crista terminalis; IAS, interatrial septum; RAA and LAA, RA and LA appendage; and SVC, superior vena cava. B, Sections from the RA and LA, illustrating endocardial trabeculae connecting to the epicardial layer. C, Epicardial and endocardial view of part of the LA free wall (detail of preparation in A, colors inverted for clarity). The epicardial layer is formed by the final branches of the endocardial trabecular network, with epicardial fibers often running at right angles to larger trabeculae.
To further investigate the distribution of fibrous tissue, we distinguished between perimysial fibrosis (fibrous tissue surrounding bundles of myocytes) and endomysial fibrosis (fibrous tissue separating individual myocytes within bundles). These parameters were quantified in relation to the distance to the epicardium. Perimysial fibrosis, quantified as the distance between muscle bundles, showed a wide distribution throughout the atrial wall, without significant differences between the RA and LA and between the ST and LT groups (Figure 3B). The degree of endomysial fibrosis, quantified as the distance between myocytes within bundles, was low throughout the atrial wall in the ST LA and RA (Figure 4B), as evidenced by the close apposition of myocytes in Figure 4A. In the LT atria, there was a higher degree of endomysial fibrosis, visible as larger intermyocyte distances in Figure 4A. Interestingly, this larger degree of endomysial fibrosis was more pronounced in the outer millimeter of the LT LA and RA than in deeper layers (Figure 4B). In LT goats, the average myocyte diameter was significantly larger than in ST goats (Figure 5A). However, in contrast to the pattern of endomysial fibrosis, the myocyte diameter was homogeneously larger throughout the atrial wall in LT goats (Figure 5B).

Figure 6A (left panel) illustrates that the increase in endomysial fibrosis in LT goats was more pronounced in the thin epicardial layer than in the underlying trabeculae. The middle panel in figure 6A depicts that, even in areas where the epicardial layer was relatively thick, increased endomysial fibrosis was still predominantly observed in the outer 1 mm. The bar chart in the right panel provides average intermyocyte distances for endocardial trabeculae and for the outer millimeter of the epicardial layer in regions where the epicardial layer was relatively thin (≤1 mm) and relatively thick (>1 mm). The degree of endomysial fibrosis was more pronounced in the outer millimeter of the atrial wall, regardless of the local wall thickness, and less pronounced in the trabeculae.

Figure 6B summarizes the degree of perimysial and endomysial fibrosis, and myocytes diameter in total, in the outer millimeter of the atrial wall and in deeper layers (>1 mm from the epicardium). In deeper layers, myocyte diameter was increased in LT goats to the same degree as in the outer millimeter. By contrast, the degree of endomysial fibrosis was more pronounced in the outer millimeter than in deeper layers.

High-Resolution Optical Mapping of Wavefront Propagation
To delineate the consequences of structural remodeling for epicardial wavefront propagation, we performed high-resolution optical mapping during local stimulation (basic cycle length 350 ms at ×1.1 threshold) in 2×2 cm areas of ST and LT left atrial free walls. Afterward, the bundle orientation within the recorded areas was reconstructed and superimposed on activation patterns (Figure 7A). The direction of fastest propagation agreed well with the epicardial fiber orientation at the stimulus...
site (Figure 7B). Figure 7C shows examples of waves in 2 ST and 2 LT LA. In the ST LA, epicardial wavefronts spread rapidly with some degree of anisotropy. In the LT LA, wavefronts expanded more slowly and had a more irregular shape. Figure 7D shows the characteristics of epicardial wavefront expansion. The size of waves increased significantly more slowly in LT LA than ST LA (left panel). As the wavefronts spread out and encountered different fiber orientations, the degree of anisotropy decreased in both ST and LT LA (middle panel). However, the degree of anisotropy was higher in LT LA than in ST LA in the first milliseconds of wavefront propagation. In ST LA, the wavefront was also more circular than in LT LA (right panel), reflecting the more irregular shape of wavefronts in LT goats.

**Effect of Endomysial Fibrosis in a Dual-Layer Atrial Model**

To investigate whether increased endomysial fibrosis in the epicardial layer can explain the more complex fibrillation patterns, and in particular, the increased incidence of EBs, we developed a simplified proof-of-principle computer model consisting of an interconnected epicardial and endocardial layer (Figure 8A). In this model, endomysial fibrosis (simulated by removing 2 out of 3 transverse connections at random in the epicardial layer) significantly increased the number of simultaneous fibrillation waves and the incidence of EBs, paralleling the observed differences between ST and LT goats in epicardial mapping experiments.

**Discussion**

In the goat model used in this study, AF leads to atrial electrical and structural remodeling, both of which are thought to make AF more stable. Electrical remodeling (a shortening of the action potential duration and effective refractory period) takes places within the first 1 to 3 days of AF. In this early phase, induced AF episode durations increase from seconds to minutes. In the ensuing months, AF stability further increases, in parallel with the slow process of structural remodeling. AF can still be cardioverted pharmacologically after 3 weeks (persistent AF), but not after 6 months (permanent AF). Here, we have used these time points to investigate how the transition from persistent to permanent AF is related to structural remodeling.

We have previously reported that AF in LT goats was characterized by a higher number of simultaneous waves (both peripheral waves and EBs) and a larger incidence of conduction block, leading to a more dissociated and complex fibrillation pattern compared with AF in ST goats. Our analysis of fibrillation patterns in this study shows that LT goats had less clustering, and thus more homogeneity in the spatial distribution of the entry points of peripheral waves and EBs, and of the occurrence of slow conduction. The combination of a higher degree of complexity and lower degree of clustering indicates an underlying process causing changes in conduction that is more widespread and homogeneously distributed in LT goats than in ST goats. The lower degree of clustering of entry points into the
recorded area (both of peripheral waves and EBs) also suggests that the possible contribution of localized high-frequency sources outside the recorded area is smaller in LT than in ST goats. These data are in agreement with several studies in patients showing that, compared with acute and paroxysmal AF, conduction patterns in longstanding persistent AF are more complex and conduction defects more widespread.6–9 The observations that paroxysmal AF is often driven by sources in the pulmonary vein region, but that in longstanding persistent AF the entire atria contribute to the AF substrate, may explain the lower efficacy of ablation strategies in longstanding persistent AF patients.11,18

To characterize the pattern of structural remodeling within the complex anatomy of the atria, we quantified the transmural distribution of fibrous tissue and myocyte diameters. We did not observe systematic differences between the appendages, trabeculated anterior regions, and nontrabeculated posterior regions of the atrial free walls (data not shown). Myocyte hypertrophy was observed throughout the atrial wall and may have contributed to altered conduction. Based on detailed models of atrial tissue structure, Spach et al have proposed that myocyte hypertrophy can lead to more pronounced propagation delays during transverse propagation.14

The difference in the transmural distributions of endomysial fibrosis and myocyte hypertrophy suggests that these 2 processes are caused by different stimuli (eg, stretch versus neurohumoral activation). For example, it is conceivable that the thin epicardial layer is subjected to a larger degree of stretch than the underlying network of trabeculae, providing a larger stimulus for reactive fibrosis in the epicardial layer.

The endomysial pattern of fibrosis we have observed, is thought to arise in reaction to certain stimuli, for example, stretch and pressure overload in the absence of myocyte death, as opposed to replacement fibrosis, which occurs as a repair mechanism after myocyte death.19 Indeed, in the goat AF model, myocytes undergo dedifferentiation without an increase in apoptosis.30 There are some interesting differences with the nature and distribution of structural remodeling in another well-established animal model of AF, the canine model of congestive heart failure (CHF) because of rapid ventricular pacing. In CHF dogs compared with control dogs, a large increase was observed in the area fraction of fibrous tissue in the atria, as quantified from Sirius Red stainings.21 By contrast, the LT group in our study did not have a larger area fraction of fibrous tissue than the ST goats, as quantified in Toluidine blue stainings. Although different stainings were used in these studies, the methods for quantification are similar. In addition, other studies on animal models of AF because of rapid atrial pacing have also shown little or no increase in the area fraction of fibrous tissue.21,22

Histological analysis in canine CHF model showed large areas of fibrosis after 5 weeks of ventricular pacing, after an early phase of necrosis and apoptosis (within 24 hours after the start of pacing).23 Thus, the pattern of fibrosis in CHF dogs is highly suggestive of replacement fibrosis after earlier damage to the atrial myocardium, as opposed to the reactive fibrosis
pattern in LT goats. Interestingly, the pattern of fibrillatory conduction in CHF model is relatively simple, and consistent with macro-reentry, as opposed to the complex fibrillation patterns observed in LT goats. Although a direct comparison is not straightforward because of the different species and other confounding factors, this supports the notion that the exact type and distribution of fibrous tissue is at least as important as the overall degree of fibrosis.

An increase in interstitial fibrosis is also seen during normal aging, which may, in part, explain why age is a major independent risk factor for AF. In a sense, AF may cause accelerated aging of the atrial myocardium. Spach et al have demonstrated that this type of microfibrosis leads to discontinuous transverse propagation on a microscopic scale because of a loss of side-to-side connections between myocytes (reviewed in Reference 13). The resulting nonuniform anisotropy allows for reentry to occur in small circuits. These studies were performed in isolated bundles or atrial regions with a highly organized fiber orientation, whereas the atrial free walls display more variability. This is reflected in the pattern of wavefront spread from a point source in our study, which showed high anisotropy close to the site of stimulation that decreased as the wavefront spread out and encountered a variety of fiber orientations. The differences between ST and LT goats in wavefront expansion are consistent with a loss of side-to-side connections because of endomysial fibrosis in LT goats. In optical mapping recordings, we have only investigated propagation during slow pacing to assess the effect of structural remodeling with fully recovered sodium current availability. Conduction heterogeneity is likely to be more pronounced at shorter intervals, with incomplete recovery of the sodium current (eg, during extrastimulation or AF).

In LT goats, endomyocardial fibrosis occurred predominantly in the thin epicardial layer, and may therefore have specific effects on propagation within the complex atrial anatomy. Scheussler et al showed in isolated canine atria that the epicardium and endocardium can show regional differences in activation time and that reentry pathways may involve the transmural plane. In a study on isolated sheep right atria, Berenfeld et al have shown how the endocardial network of trabeculae can increase the complexity of fibrillation patterns. Houben et al have proposed that with a loss of the leading role of the thin epicardial layer, the underlying trabecular network may become dominant, resulting in a more disorganized and stable type of AF. This is in agreement with the higher incidence of EBs because of transmural conduction that has been observed in patients with permanent (or longstanding persistent) AF. Using simultaneous epicardial and endocardial mapping, we have recently shown that the degree of epicardial–endocardial dissociation of fibrillation waves is substantially larger in LT goats than in ST goats. The endomyocardial fibrosis observed in the present study is likely to lead to a loss of continuity of the thin epicardial layer. This would reduce its synchronizing effect on fibrillatory propagation in the underlying trabecular network, increasing the tortuosity of reentrant pathways.
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and creating a more 3-dimensional substrate for AF. This hypothesis was strengthened by simulations using a simplified model of 2 interconnected layers, where transverse uncoupling in the epicardial layer caused an increase in 3-dimensional conduction, as evidenced by the increased incidence of EBs.

Limitations and Clinical Relevance

The goat model represents AF without preexisting structural heart disease. By contrast, a majority of patients are of advanced age or have some form of structural heart disease that leads to a substrate for AF (often including atrial fibrosis) before the

Figure 7. Difference in wavefront expansion between the short-term (ST) and long-term (LT) LA. A, Left panels: LA recording location from an LT goat, excised and back-lit to visualize the epicardial fiber orientation (epi) and the endocardial trabeculae (endo, mirrored). Right panels: reconstructed bundle orientations (yellow for epi, blue for endo) from the recording location superimposed on frames taken 5 ms after stimulation at the sites indicated by asterisks. B, For each stimulation site, the difference in angle between the reconstructed epicardial bundle orientation and the orientation of fastest wavefront spread (obtained by fitting the wavefront with an ellipse) was determined. Histograms consist of data for all stimulation sites in all goats. For both the ST and LT group, there was a good correlation between the reconstructed bundle orientation and the direction of fastest wavefront spread. C, Examples of frames taken 5 ms after stimulation in 2 ST goats (left panels) and 2 LT goats (right panels). In LT goats, wavefronts had a more irregular shape. D, Wavefront characteristics as a function of time after stimulation: total wave size (left panel), the anisotropy ratio (middle panel, calculated as the major axis/minor axis of an ellipse fitted to the shape of the wavefront), and wave circularity ($4\pi \times \text{area/perimeter}^2$; right panel). Bars represent 95% confidence intervals. In the LT LA, wavefront spread was slower, more anisotropic in the first milliseconds, and more irregularly shaped.
first onset of AF. However, the goat model allows study of the consequences of AF itself. It is conceivable that AF, even with a preexisting substrate, causes specific structural changes (e.g., endomysial fibrosis) that gradually increase AF stability. Prevention of these changes may form an important target for treatment strategies to maintain sinus rhythm. The circumstances leading to endomysial fibrosis differ from those leading to replacement fibrosis. Therefore, the underlying signaling pathways are likely to differ, which may allow specific upstream therapy.

The dual-layer mathematical model does not represent the full structural complexity of the atrial anatomy, and conduction patterns in a more complex model are likely to be quantitatively different. However, it was intended as a proof-of-principle model to test the hypothesis that transverse uncoupling in the epicardium will lead to a more 3-dimensional conduction pattern, as reflected by an increased incidence of EBs.

We propose that structural changes in LT goats, most notably endomysial fibrosis in the epicardial layer, are responsible for the increased complexity of fibrillatory conduction. We cannot directly prove a causal relation, in part, because of the difference in scale between changes in structure (micrometers) and behavior of fibrillation waves (millimeters to centimeters). Nevertheless, we propose that we present strong evidence for this relation because of the following reasons: (1) Both the alterations in structure and conduction are ubiquitously and homogeneously present in the epicardial layer. (2) Our results are in agreement with relation between endomysial fibrosis and impaired transverse propagation in detailed studies on isolated atrial muscle bundles. (3) The results of high-resolution optical mapping recordings are consistent with transverse uncoupling of myocyte fibers. (4) Changes in epicardial conduction were reproduced in a simple model of 2 interconnected layers. Taken together, this evidence suggests that specific inhibition of endomysial fibrosis may represent a promising target to prevent the progressive stabilization of AF.

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Disclosures
None.

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CLINICAL PERSPECTIVE
In many patients, atrial fibrillation (AF) becomes more stable over time and restoration of sinus rhythm correspondingly more difficult. This phenomenon is also observed in a goat model of AF, in which we have previously demonstrated the association between structural remodelling, complexity of fibrillatory conduction, and AF stability. Here, we have studied the spatial distribution of fibrillation waves and of structural changes in the atrial free walls of goats with persistent and permanent AF. Although fibrillation patterns were more complex in permanent AF, the spatial distribution of fibrillation waves and conduction abnormalities was more homogeneous. This indicates a ubiquitous remodeling process that increases the contribution of the free walls to the substrate for AF. In histological analyses, myocyte hypertrophy was observed throughout the atrial walls in permanent AF. The overall amount of fibrous tissue did not differ significantly between persistent and permanent AF. However, goats with permanent AF showed a markedly larger degree of endomysial fibrosis in the outer millimeter of the atrial myocardium. The resulting reduction in transverse electrical coupling in the epicardial layer was supported by optical mapping recordings. Based also on simulations with a proof-of-principle computer model, we propose that endomysial fibrosis reduces the synchronizing effect of the thin epicardial layer, resulting in a more 3-dimensional and stable fibrillation pattern. Prevention of endomysial fibrosis in AF patients may therefore prevent the progression of AF.
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Supplemental Material
SUPPLEMENTAL METHODS

**Animal model**
Ten ST and 7 LT goats were included in this study (weight ST, 50±3kg; weight LT, 49±4kg). All animal procedures were in accordance with national and institutional guidelines. Anesthesia was induced with 10mg/kg pentobarbital and maintained with 2% isoflurane. A bipolar screw-in J-lead (Medtronic, Minneapolis, MN) was inserted through the jugular vein into the RA and connected to an Itrel pacemaker (Medtronic) in a subcutaneous pocket in the neck. AF was maintained by applying a 1-second burst of 50Hz every 2 seconds for 3 weeks (ST) or 6 months (LT).

An initial analysis of these same ST and LT goats has been published before. In short, we have shown that the efficacy of cardioversion with a combination of AVE0118 and dofetilide was 80% after 1 month of AF and 0% after 6 months of AF. Thus, the time points of sacrifice experiments in the ST and LT group reflects the stages of persistent and permanent AF, respectively. All goats had preserved cardiac output and none showed signs of heart failure.

**Sacrifice experiments**
After 3 weeks (ST) or 6 months (LT) of AF, an open-chest follow-up experiment was performed. Anesthesia was induced with thiopental (20mg/kg) and maintained with 2% isoflurane. After left-sided thoracotomy, electrograms during AF were recorded from the LA and RA free walls using a round, high-density electrode array of 4cm in diameter, consisting of 234 unipolar recording electrodes with an interelectrode distance of 2.4mm, connected to a 256-channel amplifier (sampling rate 1kHz; filtering bandwidth 0.5-500Hz).

**Analysis of fibrillation patterns**
A detailed description of the analysis of fibrillation waves has been provided previously. Here, we have analyzed the spatial distribution of peripheral waves (i.e. waves entering the recording area from the periphery of the array), epicardial breakthroughs (i.e. waves appearing within the recording area away from the periphery), AFCLs (average interval between activations) and slow conduction (conduction times >8ms between adjacent electrodes, corresponding to a conduction velocity <30cm/s). For the dispersion patterns in AFCL, all electrodes were included, for epicardial breakthroughs and block, all electrodes except for the periphery, and for peripheral waves, only electrodes at the periphery. The degree of clustering in these parameters was evaluated by calculating the Morisita Index (higher values mean more clustering) using PASSaGE spatial statistics software.

**Optical mapping**
For optical mapping experiments, separate groups of ST (n=8) and LT (n=6) goats were used. After opening the chest, hearts were excised and perfused with cold cardioplegic solution. Cannulas were inserted into the coronary ostia and fixated with purse-string sutures through the aortic wall. The heart was then transferred to a tissue chamber and perfused at 37°C with modified Krebs-Henseleit solution (concentrations in mM: NaCl 118.5, KCl 3, NaHCO3 25, KH2PO4 1.2, MgSO4 1.2, glucose 11, CaCl2 1.2, bubbled with 95% O2 and 5% CO2, pH 7.4). A bolus of the voltage-sensitive dye di-4-ANEPPS was added and filtered solution containing 10 µM of the excitation-contraction uncoupler blebbistatin was recirculated through the heart at a perfusion pressure of 100-120cm H2O. An area of approximately 2x2cm of the LA free wall was illuminated with Luxeon-I power LEDs (505nm, Philips LumiLEDs) and imaged with a 100x100 pixel Ultima-L CMOS camera (SciMedia USA Ltd.), providing a spatial...
resolution of approximately 200µm. A unipolar stimulation electrode was placed at various sites within the field of view (range 6-12 sites per recording location, average 9±1), with a reference electrode in the LV cavity. Imaging files were recorded at a sampling rate of 2kHz during pacing at an output of 1.1x threshold and a basic cycle length of 350ms. Data were analyzed offline using custom made software. For the analysis of wavefront propagation, fluorescence movies were imported in ImageJ and the wave size and circularity \((4\pi\times(area/perimeter^2))\) was determined for each millisecond using the 'particle-analysis' function. The wavefronts were fitted with an ellipse function, and the degree and direction of anisotropy was quantified as the ratio of major to minor ellipse axis and the angle of the major axis, respectively.

After the optical recordings, the corners of the fields of view were marked and the recording location was excised, stretched to visualize the relation between the epicardial layer and endocardial bundles and photographed with back-illumination. The photographs were then transformed back to a square area with the dimensions of the field of view in the preparation using the 'puppet-warp' function in Adobe Photoshop (Adobe Systems Inc.). The epicardial and endocardial bundle orientation was reconstructed from these images and projected on the recorded wavefronts to correlate wavefront propagation to bundle orientation.

**Histological analysis**

After the electrophysiological measurements, the complete atria were fixed in zinc-acetate buffered formalin solution (4%, pH 5.5) in 5 ST and 5 LT goats. The entire RA and LA free walls (including the appendages) were removed from the remaining atrium and embedded in Technovit-7100 plastic resin (Heraeus-Kulzer). Transverse sections of 5µm were produced in parallel to the anterior-posterior axis with a macrotome and stained with toluidine-blue in order to distinguish myocytes (dark blue) from connective tissue (light blue). For quantitative analysis, several areas of both atria were examined in the region midway between the AV ring and the interatrial septum and spanning from the anterior side of the appendage to the posterior side of free walls. Photographs were taken at low magnification (25x) to count the incidence of larger interruptions of the epicardial layer (distances >0.5mm devoid of myocytes in the epicardial layer) and at high magnification (200x) to determine the myocyte diameter at the level of the nucleus. In addition, thicknesses of perimysial fibrous tissue (defined as the distance between bundles of myocytes) and endomysial fibrous tissue (defined as the distance between myocytes within bundles) were determined. For each individual measurement of these parameters, the local distance to the epicardium (excluding the epicardial fibrous layer) was determined. Atrial anatomy was studied in 6 separate hearts from goats used in other experiments.

**SUPPLEMENTAL DATA**

**Supplemental figure 1: distribution of AF cycle lengths**

**Methods.** The average AF cycle length (AFCL) was determined for each electrode from the intervals between activation time points (maximal negative dV/dt). To quantify the dispersion in activation frequency, Morisita indices were calculated for the spatial distribution of the number of activations per seconds.

**Results.** Figure s1A shows examples of the AFCL distribution in three different ST and LT left atria, with the corresponding Morisita index in the lower right corner of each map. The maps of the LT goats show a higher degree of heterogeneity in AFCL, due to areas with a relatively long AFCL (red areas in the 1st and 3rd LT map). The Morisita index was
significantly higher in LT vs. ST goats for both atria. The AFCL was not significantly different between the ST and LT group for either atrium. Connected points in figure s1B show the average AFCL for the RA and LA in each individual goat included in the study. The RA had a shorter AFCL in 5 out of 10 ST goats and in 5 out of 7 LT goats.

**Supplemental figure 2: overall degree of fibrosis**

**Methods.** To quantify the total area fraction occupied by fibrous tissue, low-magnification photos (25x) were imported in Adobe Photoshop (Adobe Systems Inc.) and the epicardial, endocardial and perivascular fibrous tissue was selected using the 'quick selection' tool and removed (figure s2A, step 1). The processed photographs were then imported in ImageJ and a threshold was set to distinguish fibrous tissue (light) from myocytes (dark), as illustrated in figure s2A, step 2. The area fraction of fibrous tissue was calculated as the ratio of fibrous area/total tissue area.

**Results.** The total area fraction occupied by fibrous tissue was not significantly different between the ST and LT group for either atrium (figure s2B).

**Supplemental figure 3: determination of perimysial and endomysial fibrosis**

**Methods.** Figure s3 shows examples of the determination of the distribution of perimysial fibrosis (separating bundles of myocytes) and endomysial fibrosis (separating myocytes within bundles). Figure s3A shows examples for an ST and LT left atrium of the distribution of muscle bundles (outlines in red) in sections of the atrial wall stained with toluidine blue. Examples of determinations of inter-bundle distances (perimysial fibrosis) are indicated by yellow lines. Figure s3B shows an example at higher magnification of a bundle in an LT left atrium (before and after conversion to grayscale). In ImageJ, profile plots were made of the intensity across cell-to-cell boundaries, resulting in low intensity troughs for myocytes and high intensity peaks for extracellular space (ECM). The points at half-maximal intensity of each peak were taken as the transition from myocyte to ECM, in order to determine inter-myocyte distances (endomysial fibrosis) and myocyte diameter.

**Results.** See main text.

**Supplemental movies: epi-endocardial connections**

**Methods.** The movie clips show a reconstruction of the RA wall (movie 1) and LA wall (movie 2). Toluidine-stained serial tissue sections were photographed at low magnification and aligned using the 'Align Slices - rigid body' algorithm in ImageJ, in a version with the MBF macro collection installed (available at www.macbiophotonics.ca/imagej).

**Results.** In the movie clips, various contact points with the epicardial layer can be seen along the length of trabecular muscle bundles. In this sense, the trabeculated parts of the atrial wall have an umbrella-like structure rather than a parachute-like structure.

**Supplemental methods: Description of the dual-layer mathematical model**

To investigate the effect of a reduction in transverse electrical coupling in the epicardial layer on the complexity of AF and the incidence of epicardial breakthroughs, a novel proof-of-principle computer model was developed. Atrial tissue was described by a monodomain reaction-diffusion model comprising two layers of 4 x 4 cm. Each of the layers was composed of 400 × 400 segments of size 0.01 × 0.01 cm and atrial membrane behavior for each segment was based on the Courtemanche-Ramirez-Nattel model. Electrical connections between the
two layers were incorporated by adding resistances between opposing segments in a circular area with radius 0.1cm (conductivity $\sigma z=0.5\text{mS/cm}$). These connections could be introduced or removed at any time during the simulation. The monodomain model is described by

$$\chi \left( C_m \frac{dV_m}{dt} + I_{\text{ion}} + I_{\text{stim}} \right) = -\nabla \cdot (G \nabla V_m),$$

where $\chi$ is membrane surface-to-volume ratio, $C_m$ is membrane capacitance, $I_{\text{ion}}$ is ionic membrane current, $I_{\text{stim}}$ is externally applied stimulus current, and $G$ is the conductivity tensor. In the present study, $\chi=2000\text{cm}^{-1}$, $C_m=1\mu\text{F/cm}^2$. In the isotropic endocardial layer, conductivities were the same in both directions, $\sigma_x=\sigma_y=0.5\text{mS/cm}$. In the anisotropic epicardial layer, $\sigma_x=1.0\text{mS/cm}$ and $\sigma_y=0.12\text{mS/cm}$. To simulate endomysial fibrosis, 2 out of 3 transverse connections between segments were removed at random. All simulations were performed using ionic membrane currents reflecting complete electrical remodeling, i.e. maximum conductivities for transient outward potassium current ($I_{\text{to}}$) and L-type calcium current ($I_{\text{Ca}L}$) were reduced by 60 and 65%, respectively, and maximum conductivity for inward rectifier potassium current ($I_{\text{K1}}$) was increased with 100%.

**Simulation protocol.** To investigate the effect of endomysial fibrosis on fibrillatory behavior and stability of AF episodes the following simulation protocol was applied. In one of the layers, a spiral wave was initiated using an S1-S2 protocol, while the other layer was not stimulated. The situation one second after the start of the simulation was used as the starting condition for an additional 6 seconds of simulation time in which the layers were connected by 12 connections. To exclude a bias caused by a particular geometry of the connection points, 8 separate simulations were performed with different sets of 12 randomly chosen connections. Connection points for each simulation were chosen such that two connections were at least 0.2cm apart. All simulations were continued for 6 more seconds, either without or with simulated endomysial fibrosis in the epicardial layer.

**Analysis.** AF complexity (the number of waves and epicardial breakthroughs) was analyzed for all simulations. A wave was defined as a contiguous area in which all segments have membrane potentials above the excitation threshold of -60mV. The number of waves was calculated for each millisecond during the entire simulation time. An epicardial breakthrough (BT), a wave that appears in the epicardial layer and cannot be linked directly to the propagation of other waves in the epicardial layer, was detected as follows. Areas containing connection points were monitored each 1ms. If a new wave appeared in one of these areas and could not be related to the propagation of other waves in that layer and if it had a size at most the size of the connection area, it was marked as a candidate breakthrough. If a candidate breakthrough increased in size within the next 2ms, it was counted as a breakthrough.

**Numerical methods and implementation.** The model used for the present simulation study was based on our previously published bi-domain model. The mono-domain equation was solved assuming no-flux boundary conditions using an explicit numerical scheme with time steps of 0.01ms as previously described. Gating variables and intracellular ion concentrations were updated with time steps of 0.01ms during the action potential upstroke and otherwise with time steps of 0.1ms. Gating variables were integrated using the Rush-Larsen method. The model was implemented in C++ and executed on a normal PC with Intel i7 processor and 6GB memory. It took 72 hours to simulate an AF episode of 6 s on the double-layer model. Up to six simulations could run simultaneously on the multi-core processor without increase in computation time.
**Statistical analysis.** Statistical tests were performed to compare the two groups of simulations (dual-layer without fibrosis n=8 and dual-layer with fibrosis n=8). For each group, the average number of waves and the average number of BTs during the whole simulation time was calculated. Each data set was tested for normal distribution using the Kolmogorov-Smirnov test. An unpaired Student t-test was performed to compare normally distributed data sets.

**References**

Figure Suppl. 1
A

muscle tissue  

fibrous tissue

step 1

step 2

B

fibrous tissue % of total area

LA  RA

Figure Suppl. 2
**Figure Suppl. 3**

A

Toluidine Blue converted to grayscale

B

profile plot

ST LT