Active stiffening of mitral valve leaflets in the beating heart

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Mitral valve (MV) disease affects millions worldwide. Medical and surgical therapies for this disease have traditionally assumed that the valve leaflets, the key components of the valve, are passive flaps (46). However, muscle fibers (2, 6, 11, 12, 18, 24, 37, 39), blood vessels (6, 8, 11, 24, 37, 39), and nerves (1, 6–13, 18–20, 26, 29, 35, 36, 39, 43–45) have been recognized in the MV leaflets of animals and humans since the 19th century, and the physiological function of these entities, along with other contractile elements in the leaflets, such as smooth muscle (8, 18, 26, 47) and valvular interstitial cells (VICs) (4, 13, 18, 23, 26, 28, 38, 40), is not understood.

Leaflet elastic modulus response to these interventions (circumferential and radial; now including IVC and IVR) from biplane radiography of markers sewn to the components of the ovine MV. These values were obtained before (CTRL_ESML) and after reversible blockade of β-receptors with intravenous esmolol (ESML) and before (CTRL_STIM) and after stimulation of the region of aortic-mitral continuity (STIM) with subthreshold electrical pulses.

MATERIALS AND METHODS

Experimental animals. We previously reported baseline leaflet $E_{\text{circ}}$ and $E_{\text{rad}}$ during IVR in a cohort of 17 sheep (21). By protocol, after we acquired these initial baseline data, we conducted two intervention studies (ESML and STIM), with each intervention preceded by its own control (CTRL_ESML and CTRL_STIM). We report here the leaflet elastic modulus response to these interventions (circumferential and radial) from biplane radiography.

All animals received humane care in compliance with the “Principles of Laboratory Animal Care” formulated by the National Society for Medical Research and also in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, Revised 1985). This study was approved by the Stanford Medical Center Laboratory Animal Review Committee, which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International, and was conducted according to Stanford University policy.

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Surgical preparation. The sheep (54 ± 8 kg) were premedicated with ketamine (25 mg/kg im) for venous and arterial catheter placement, anesthetized with inhalational isoflurane (1.0–2.5%), intubated, and mechanically ventilated. Once normal cardiac function and valve competency were confirmed with epicardial echocardiography through a left thoracotomy, 13 miniature radiopaque tantalum markers were surgically implanted into the subepicardium to silhouette the LV chamber along four equally spaced longitudinal meridians (ventricular markers, Fig. 1A). On CPB, a total of 35 radiopaque tantalum markers were sewn to the following sites: 1 at the tip of each papillary muscle [anterior and posterior papillary muscle (APM and PPM), Fig. 1A], 16 around the mitral annulus (annular markers, Fig. 1A), 16 on the atrial aspect of the anterior MV leaflet (markers 1–7, Fig. 1B) and 9 on the leaflet belly (markers 8–16, Fig. 1B), and 1 on the central edge of the middle scallop of the posterior MV leaflet (Fig. 1A). A single tantalum loop (0.6 mm ID, 1.1 mm OD, 3.2 mg each) was used for each leaflet marker (Fig. 1C). After marker implantation, a pacing wire electrode was placed in the region of aortic-mitral continuity (near marker 20, STIM, Fig. 1B) and exteriorized. The animals were weaned from CPB, and a micromanometer pressure transducer (model PA4.5-X6, Konigsberg Instruments, Pasadena, CA) was placed in the LV chamber through the LA and exteriorized.

Data acquisition. The animals were transferred to the experimental catheterization laboratory and placed in the right lateral decubitus position for acquisition of data under open-chest conditions. Two micromanometer pressure transducers (model MPC-500, Millar Instruments, Houston, TX) were calibrated and introduced via the carotid artery to measure LVP and aortic pressure (AoP) immediately distal to the aortic valve. The Konigsberg pressure transducer was calibrated (by linear regression of Millar and Konigsberg signals) against the two Millar pressure transducers while all transducers were in the LV, then the Konigsberg was pulled back into the LA for measurement of LAP.

Videofluoroscopic images (60 frames/s) of all radiopaque markers were acquired in multiple-beat runs using a biplane videofluoroscopy system (Philips Medical Systems, Pleasanton, CA) with the heart in normal sinus rhythm and ventilation transiently arrested at end expiration. These images were obtained before (CTRL_ESML) and after (ESML) β-blockade with esmolol (10–25 mg bolus to achieve ~15% reduction in LVP) and then before (CTRL_STIM) and after (STIM) subthreshold pacing via the pacing electrode for ~10 s (1–3 mA, 320/min). Marker coordinates from each view were then merged to yield the three-dimensional coordinates of the centroid of each marker in each frame. LVP, LAP, AoP, and ECG voltage signals were digitally recorded simultaneously during marker data acquisition and synchronized with the images. After data acquisition, the animals were euthanized, and the hearts were excised. Ex vivo photographs of the MV and subvalvular apparatus were used to identify the chordae tendineae insertion points on the MV leaflet.

Fig. 1. A: radiopaque marker sites on left ventricle (LV), mitral annulus, and leaflets. AML and PML, anterior and posterior mitral valve (MV) leaflet; APM and PPM, anterior and posterior papillary muscle. B: radiopaque marker sites on the MV anterior leaflet, with circumferential (C) and radial (R) axes and subthreshold electrical pulse stimulation site (STIM). C: intraoperative photograph of markers sewn to the MV anterior leaflet. D: representative LV pressure (LVP), left atrial pressure (LAP), and interleaflet distance (D) data showing LVP interval during which isovolumic contraction (IVC) stiffness is measured (red dots) and anterior leaflet shape at the end of IVC (top left) and pressure-matched LVP interval during which isovolumic relaxation (IVR) stiffness is measured (blue dots) and anterior leaflet shape at the beginning of IVR (top right). Leaflet vertical dimension is color-coded from −3 to +4 mm to illustrate leaflet shape.
ological range of pressures in these beating hearts. Two regions (PSHELL in Hypermesh) were individually developed for IVC and IVR intervals for the anterior leaflet belly marker positions. The Optistruct solver was defined normal to the radial axis ($R$, Fig. 1B). The leaflet circumferential axis ($C$, Fig. 1B) was defined as the leaflet and in the plane containing $R$ and the posterior commissural marker (marker 23, Fig. 1, A and B).

The material model of the leaflet was assumed to be orthotropic linear elastic (MAT8 in Hypermesh), an assumption supported by our analysis (unpublished observations) showing that the stress-strain relationship of these leaflets is linear over the physiological range of pressures in these beating hearts. Two regions (PSHELL in Hypermesh) were used to define the varying thickness of the leaflet with use of thickness data obtained from our histological study of an anterior leaflet from a representative ovine heart. The first defined a region from the annulus to 75% of the leaflet toward the free edge; this region had thickness values that varied linearly from 1.2 mm at the annulus to 0.7 mm 75% toward the leaflet free edge. The second region defined the remaining 25% of the leaflet with a uniform thickness of 0.2 mm.

The bicubic surface fit of the MV leaflet was then meshed with plane-stress quadrilateral shell elements. A typical anterior leaflet was meshed with 2,200 elements, yielding an element size of 0.004 cm$^2$. This mesh allowed experimental displacements to be matched with <5% root-mean-square error for the final material properties obtained.

The strut chordae were defined as ropelike structures undergoing pure tension (MAT1 in Hypermesh). A previously published ex vivo material properties of the anterior MV leaflet belly during IVC and IVR. In this algorithm, the model-simulated displacements of the nine leaflet belly markers (markers 8–16, Fig. 1B) from each iteration were compared with the actual measured displacements of these nine markers during IVC or IVR to yield an objective function defined as the root-mean-squared displacement difference between measured and actual displacements of the nine leaflet belly markers.

Statistical analysis. Values are group means ± SD. Three-beat averages were used to characterize the data for each animal. Data were compared using one- and two-way repeated-measures ANOVA with Bonferroni’s post hoc test (Sigmastat 3.5, Systat Software, San Jose, CA). Statistical significance was set at $P < 0.05$ unless otherwise specified.

RESULTS

Table 1 shows the hemodynamic and annular dimensional response to the ESML and STIM interventions. Relative to

Table 1. Hemodynamics and annular dimensions

<table>
<thead>
<tr>
<th></th>
<th>CTRL_ESML</th>
<th>ESML</th>
<th>CTRL_STIM</th>
<th>STIM</th>
</tr>
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<tbody>
<tr>
<td>Heart rate, min$^{-1}$</td>
<td>88±13</td>
<td>83±11*</td>
<td>84±11*</td>
<td>83±11*</td>
</tr>
<tr>
<td>LV ESV, ml</td>
<td>100±16</td>
<td>104±18*</td>
<td>104±17*</td>
<td>104±18*</td>
</tr>
<tr>
<td>LV SV, ml</td>
<td>26±6</td>
<td>23±7*</td>
<td>24±6</td>
<td>24±6</td>
</tr>
<tr>
<td>LV EDP, mmHg</td>
<td>14±3</td>
<td>15±3</td>
<td>15±3</td>
<td>16±3</td>
</tr>
<tr>
<td>LV ESP, mmHg</td>
<td>84±8</td>
<td>71±12</td>
<td>74±9*</td>
<td>71±9*</td>
</tr>
<tr>
<td>S-L dimension at ES, cm</td>
<td>2.5±0.2</td>
<td>2.6±0.2</td>
<td>2.5±0.2</td>
<td>2.5±0.2</td>
</tr>
<tr>
<td>C-C dimension at ES, cm</td>
<td>3.5±0.3</td>
<td>3.6±0.3*</td>
<td>3.6±0.3*</td>
<td>3.6±0.3*</td>
</tr>
<tr>
<td>Total annular perimeter at ES, cm</td>
<td>10.8±0.7</td>
<td>11.0±0.7*</td>
<td>11.1±0.7</td>
<td>11.0±0.7</td>
</tr>
<tr>
<td>Mitral annular area at ES, cm$^2$</td>
<td>8.1±1.1</td>
<td>8.5±1.0*</td>
<td>8.6±1.3*</td>
<td>8.5±1.3*</td>
</tr>
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</table>

Values are group means ± SD ($n=10$). LV, left ventricular; ESV, end-systolic volume; SV, stroke volume; EDP, end-diastolic pressure; ESP, end-systolic pressure; S-L, septal-lateral; C-C, commissure-commissure; ES, end systole. Control data (CTRL_ESML and CTRL_STIM) were obtained immediately before esmolol (ESML) and subthreshold electrical pulse stimulation (STIM) interventions, respectively. *$P$ ≤ 0.001 vs. CTRL_ESML [by 1-way repeated-measures ANOVA with Bonferroni’s post hoc test (level of significance adjusted to $P < 0.05/4$)].
CTRL_ESML, ESML β-blockade reduced heart rate, stroke volume, and LV end-systolic pressure; increased LV end-systolic volume and mitral annular comissure-commissure and perimeter dimensions and area; but did not change LV end-diastolic pressure. From CTRL_STIM to STIM, these values were unchanged, inasmuch as the STIM pulses were intentionally subthreshold. Differences between CTRL_STIM and CTRL_ESML likely result from CTRL_STIM consistently following CTRL_ESML in the protocol.

Table 2 and Fig. 2 show the responses of leaflet moduli to the ESML and STIM interventions. [To avoid cumbersome triple-subscript notation, we identify modulus variables by column (A–H) and row (1–2) in Table 2 and Fig. 2 (e.g., A1 for CTRL_ESML IVC E_{circ}, H2 for STIM IVR E_{rad}).]

We found that 1) MV leaflet stiffness is anisotropic, with E_{circ} 2.7–3.3 times greater than E_{rad} under all conditions (mean A1 = 2.9・A2, B1 = 3.0・B2, C1 = 3.3・C2, D1 = 3.0・D2, E1 = 2.9・E2, F1 = 3.1・F2, G1 = 2.7・G2, and H1 = 2.9・H2), 2) MV leaflet stiffness changes during the cardiac cycle, with control IVC stiffness 1.5–1.7 times greater than control IVR stiffness for E_{circ} and E_{rad} (mean A1 = 1.5・B1, A2 = 1.6・B2, E1 = 1.6・F1, and E2 = 1.7・F2), and the control IVC-IVR stiffness difference was anisotropic, with the circumferential difference two to three times that of the radial difference ([(A1 - B1) = 2.6・(A2 - B2)] and [(D1 - E1) = 2.7・(E2 - F2)], 3) ESML had no effect on IVR stiffness ([D1 = C1] < A1 and [B2 = C2] < A2), 4) STIM doubled IVC stiffness from CTRL ([H1 = 1.9・F1] and [H2 = 2.0・F2] but preserved the anisotropic stiffness ratio ([H1 = 2.9・H2]), and 5) STIM increased IVC stiffness in parallel with IVC stiffness ([G1 - E1] = ([H1 - E1]) and [G2 - E2] = ([H2 - F2]).

Figure 3, histological sections of an ovine anterior MV leaflet, shows the expected continuity between LA muscle and atrial muscle on the atrial side of the anterior MV leaflet (Fig. 3, A and D), scattered muscle cells and collagen fibers in the leaflet (Fig. 3, B and C), and bands of smooth muscle just beneath the atrial and ventricular leaflet surfaces (Fig. 3, E and F).

**DISCUSSION**

The results of the present study strongly support both hypotheses tested. We found 1) an increase in leaflet stiffness of the closed valve during IVR and IVC in response to subthreshold electrical stimulation of the remote, neurally rich region of aortic-mitral continuity and 2) a transient increase in MV leaflet stiffness during IVC, above IVR values, that is abolished by β-receptor blockade.

Three force-developing leaflet components are currently known that could account for these stiffening behaviors: leaflet cardiac muscle, leaflet smooth muscle, and VICs.

**Leaflet cardiac muscle.** The atrialis layer of the anterior MV leaflet contains cardiac muscle cells and well-defined cell bundles, several myofibrils thick, arranged end-to-end and side-to-side, in continuity with LA muscle, surrounded by connective tissue, accompanied by blood vessels and efferent nerves, and tapering in thickness, number, and orientational regularity from annular base to central leaflet, with only slips of muscle near the occlusive margin (2, 6, 11, 12, 18, 30, 39, 47, 48). As shown in Fig. 3, A and D, this muscle is present at this location in the ovine anterior leaflet. The ultrastructure of this cardiac muscle is similar to that of atrial muscle (12), and, similar to LA muscle, such cells contract with electrical stim-
ulation (12, 39) via propagated depolarization [35-ms stimulus latency, 210-ms refractory period, 6% shortening (39)] and exhibit a positive inotropic response to norepinephrine and tyramine and a negative inotropic response to stimulus frequency (25, 39) and acetylcholine (reversible with atropine) (12, 39).

We propose that this leaflet atrial muscle is responsible for the twitch behavior of the closing valve during IVC, but not the tone behavior of the closed valve during IVR. Previous studies demonstrated that anterior leaflet cardiac muscle is excited with each atrial systole (7, 33) and, similar to atrial myocardium (25), contracts for only \( \frac{1}{2} \) of ventricular systole (32). In isolated tissue, excitation propagates from the LA into the anterior leaflet (12, 48) but spreads more slowly in the leaflet (40–60 ms) than in the atrium (6–10 ms) (12), with force developed for ~200 ms, at most, after transmembrane depolarization of leaflet cardiac muscle (12). However, as shown in Fig. 1D, 200 ms (12 frames) corresponds to less than half of ventricular systole in these ovine hearts. Thus, as with LA muscle itself, leaflet cardiac muscle is expected to develop active force during IVC, but it is not expected to contribute to leaflet stiffness during IVR, inasmuch as it is relaxed at that time.

Leaflet smooth muscle. Anterior MV leaflets also contain smooth muscle. The atrialis layer of anterior leaflets contains smooth muscle cells and cell bundles (2–30 cells thick), located in association with blood vessels and elastic sheets and in close proximity to unmyelinated nerve terminals and observed most prominently in proximal and middle regions of the leaflet, but also in the distal third near the leaflet edge (8, 18, 26, 47). As reported by Wit et al. (47), we observed layers of smooth muscle cells just beneath the endocardial cell layers on the atrial and ventricular surfaces of the ovine anterior leaflet (Fig. 3, E and F). Smooth muscles can also respond rapidly to stimulation, as required in the present STIM study, where stiffness changed within tens of seconds after the onset of electrical stimulation. Thus smooth muscles could provide variable, multibeat leaflet tone that could be observed in the closed valve during IVR, when leaflet atrial muscle is relaxed. We do not think, however, that smooth muscle per se is responsible for the twitch or tone stiffening observed. Previous studies have shown that the maximum force-developing capability of smooth muscle ranges from 0.003 to 0.87 N/mm² (16, 17, 50) and that the elastic modulus of such muscle ranges from 0.008 to 0.36 N/mm² (17, 50). Our analysis shows that the maximum force required in the ovine anterior leaflet ranges from 1 to 4 N/mm², with \( E_{\text{circ}} \) and \( E_{\text{rad}} \) of 5–100 N/mm² (Fig. 2). Thus, even if the entire leaflet was composed of smooth muscle, it would be incapable of generating sufficient force or elastic modulus. Smooth muscle is quite sparsely distributed in these leaflets (Fig. 3); thus, although smooth muscle cannot be entirely ruled out as part of a stiffening mechanism, it fails to account for the force and moduli of the leaflet in the beating heart by one to two orders of magnitude.

Fig. 3. Muscle and collagen staining of an ovine MV anterior leaflet that was immersed in 10% buffered formalin, dehydrated, and embedded in paraffin wax blocks. LA, left atrial surface; LV, LV surface; AoV, aortic valve. Adjacent radial slices from the middle of the leaflet stained with Silverman-Movat Pentachrome (A–D: red = muscle, yellow = collagen, blue = glycosaminoglycans, black = elastin) and for smooth muscle \( \alpha \)-actin (E and F; brown = smooth muscle). Original magnification \( \times 16 \) (A and B), \( \times 50 \) (C), and \( \times 1,000 \) with oil immersion (D–F). Isolated smooth muscle cells may also be present throughout the leaflet, although they are not visible at this magnification. Pattern and distribution of structural components that comprise MV leaflets of mammals vary in different species; thus, although there are many similarities, caution must be exercised in extrapolating these results to other species. The extent to which myocytes extend from the annulus into the leaflet, roughly one-third to one-half of the distance from the mitral annulus to the leaflet edge, appears to be a common feature of human anterior MV leaflets (30) and these ovine anterior MV leaflets. Such structural measurements suggest major heterogeneity in leaflet architecture that could significantly affect regional leaflet stiffness.
VICs. Anterior MV leaflets also contain VICs, occupying large portions of the leaflet, particularly the medial and distal regions (4, 13, 18, 23, 38, 40). VICs have characteristics intermediate between fibroblasts and smooth muscle cells (13). They come in close contact with extracellular collagen and may be targets for nearby adrenergic motor nerves, the terminals of which are closely apposed (30–80 nm) (13, 26). VICs have communicating (gap) junctions, so that activity set up in one cell can readily spread to the next cell without significant delay (13). They are contractile: epinephrine and angiotensin II induce very slow, tonic contractions (likely actomyosin-based) in cultured and valvar strips (13, 38). Their collagen synthesis and smooth muscle α-actin content, and hence stiffness, may relate directly to the pressure experienced by the leaflet (28).

The contractile behavior of VICs in vitro, however, is insufficient to account for the rapid increase in stiffness observed in the present STIM study (responses observed within tens of seconds after the onset of electrical stimulation), inasmuch as VICs in vitro exhibit very slow (minutes to hours) force-developing responses to epinephrine or electrical stimuli (13, 38). However, because of their ubiquity and smooth muscle-like behavior and the possibility that VICs in vivo may have a much faster response to stimulation than VICs in vitro, they are good candidates for the stiffness observed during IVR that is increased with STIM.

**Conceputal leaflet material model.** Figure 4 provides a conceptual model consistent with the material behavior observed in the present in vivo study, as well as the findings from previous ex vivo studies (3, 14, 22, 27). The passive collagen fibers in the leaflet are represented by coiled springs, and the finding that circumferential stiffness is greater than radial stiffness in isolated leaflets (3, 14, 22, 27) results from a model-postulated greater density of circumferentially oriented than radially oriented collagen fibers. Contractile elements (red arrows, possibly VICs), in close proximity to these collagen fibers (40) and developing force in vivo, but not ex vivo, greatly increase all collagen spring constants in vivo relative to in vitro. Thus, as we reported recently (21), IVR leaflet elastic moduli are increased in vivo by orders of magnitude from ex vivo values, but the stiffness anisotropy, with circumferential stiffness much greater than radial stiffness, is maintained in the beating heart (Table 2: $B_1 = 3 \cdot B_2$ and $F_1 = 3.1 \cdot F_2$). Although circumferential and radial elements are illustrated as independent entities in Fig. 4, they may also be coupled, as discussed by May-Newman and Yin (27) and supported by the parallel circumferential and radial response to the ESML and STIM interventions in the present study.

In this model, the contractile elements (possibly VICs) associated with the collagen fibers provide the more slowly varying leaflet tone that 1) is unresponsive (or perhaps only very slowly responsive) to β-blockade, 2) is present during IVC and IVR, 3) is doubled by STIM (Table 2: $H_1 = 1.9 \cdot F_1$ and $H_2 = 2.0 \cdot F_2$), yet 4) preserves leaflet anisotropy (Table 2: $H_1 = 2.9 \cdot H_2$). The red entities at the top of Fig. 4 represent leaflet atrial muscle (Fig. 3, A and D) that is stimulated during each atrial systole, exhibits a brief (<100-ms) twitch response (abolished by β-blockade) during IVC, but is no longer contracting during IVR. In the present study, leaflet atrial muscle increased circumferential and radial leaflet stiffness transiently during IVC relative to IVR by ≥50% (Table 2: $A_1 = 1.5 \cdot B_1$, $A_2 = 1.6 \cdot B_2$, $E_1 = 1.6 \cdot F_1$, and $E_2 = 1.7 \cdot F_2$). Leaflet muscle was unresponsive to the subthreshold STIM, inasmuch as the stiffness increments ($G_1-H_1$) and ($E_1-F_1$) did not differ significantly, nor did ($G_2-H_2$) and ($E_2-F_2$); thus the significant increase in IVC stiffness with STIM appears to result from the STIM-induced increase in IVR stiffness alone.

**IVC twitch.** The anisotropy of the twitch response suggests the presence of two to three times greater force development from leaflet atrial fibers oriented circumferentially than radially during IVC [Table 2: $(A_1-B_1) = 2.6 \cdot (A_2-B_2)$ and $(E_1-F_1) = 2.7 \cdot (E_2-F_2)$], as schematically depicted in Fig. 4. This may reflect the unusual structure of leaflet atrial muscle cells that have been observed to form a loose meshwork and interweave with and cross over each other, are attached not only end-to-end, but also side-to-side, and contain myofibrils oriented in many directions (11, 18), yet with a pronounced circumferential orientation. Such a meshwork could briefly augment radial and circumferential leaflet stiffness to prevent ballooning of the cusps during IVC when the valve is suddenly subjected to high LVP. These IVC findings, coupled with the previous demonstration of a transient bending of the anterior leaflet toward the posterior leaflet that can be abolished by ablation of leaflet cardiac muscle in the open heart on CPB (7, 32, 33) and ablation of leaflet cardiac muscle that delays leaflet closure by ~35 ms in the pumping heart (41), support the concept that transient twitch contractions of leaflet atrial muscle aid valve closure.

**Neural networks.** We do not know whether the electrical pulses in the STIM study were operating directly or by paracrine mechanisms, although we favor the latter, considering the very high rate and subthreshold amplitude of the STIM pulses. There are ample efferent neural networks in the LA subendocardial plexus [the most profusely innervated area of the heart...
function must be taken into account when considering not only therapeutic approaches to MV disease, but even the definitions of MV disease itself. The improved understanding of the structure-function relationships in these native, active valves could uncover new targets for pharmacological intervention and provide important insights to improve the future design and durability of tissue-engineered MVs.

The present study also employs the most sophisticated inverse finite-element model of the MV developed to date, inasmuch as it is based, for the first time, on experimental measurements of instantaneous LVP, LAP, and AoP with the simultaneous three-dimensional positions of all the elements of the mitral complex (annulus, leaflets, and papillary muscles) throughout the cardiac cycle in the beating heart. Such a finite-element model could find potential future applications as an aid to planning patient-specific medical and surgical therapies for MV disease, taking into account stress-and-strain distributions in the valve leaflets, as well as the location and function of critical blood vessels, nerves, and multiple, neurally controlled contractile systems.

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