SUPPLEMENTAL MATERIAL

Local Application of Leptin Antagonist Attenuates Angiotensin II–Induced Ascending Aortic Aneurysm and Cardiac Remodeling

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Methods and materials

Animals:

Animal experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee, Harvard Medical School (protocol No. 05004) and compiled with the Guide for the Care and Use of Laboratory Animals (National Institute of Health Publication No. 85-23, Revised 1996).

Sixteen week-old male ApoE-deficient mice ($ApoE^{-/-}$, C57BL/6 background; Jackson Laboratory) were used. Following an acclimatization period of 7 days mice were weighed, and SBP was measured via tail cuff as described in more detail below. Each mouse underwent echocardiographic examination of the ascending aorta and heart to establish baseline anatomical and hemodynamic parameters.

Leptin application model:

A novel mouse model was used to simulate local leptin synthesis in the ascending aorta. A slow release film made of polylactic co-glycolic acid (PLGA, Fisher Scientific) matrix (1x1.5mm) containing either 2μg mouse leptin or no protein (control) was applied through left mini-thoracotomy on the anterior surface of the proximal ascending aorta in $ApoE^{-/-}$ mice (Figure 1G, Figure S3) (see methods below). This model was utilized in two separate experiments that differed by two parameters: the type of postoperative diet, and the duration of follow-up. In the first experiment, mice were fed postoperatively with either high fat diet (HFD, 60%Kcal of fat, Research Diets Inc.) or normal chow diet (NCD) postoperatively according to the experiment and were followed-up for 45 days or monitored for 30 or 60 days, respectively.

Mice underwent a left mini-thoracotomy through the third intercostal space under endotracheal general anesthesia (Isoflurane 1%). The thymus and pre-aortic fat were retracted to expose the proximal ascending aorta. A PLGA (Fisher Scientific) miniature film (1x1.5 mm) carrying 2μg leptin (mouse leptin, Sigma, L3772) was applied to the anterior surface of the ascending aorta (see intra-operative photo, Figure S3). Control mice underwent application of a PLGA film devoid of the protein. The film was secured in position by the adjacent peri-vascular tissue. The left thoracotomy wound was closed when lungs were fully inflated to prevent residual pneumothorax using interrupted 7-0 peri-costal Dexon (polyglycolic acid) stitches. Intercostal muscles were approximated with similar stiches, and the skin closed with interrupted 7-0 monofilament (Prolene) stitch. Mice were kept under surveillance for up to 60 days, during which body weight, blood pressure measurement and echocardiographic examination (see below) of the ascending aorta and heart were performed weekly or bi-weekly, including a final examination prior to euthanasia.

AngII infusion/ Local LepA application model:

All mice were similarly treated and prepared for surgery as in the leptin application model. Through an identical left mini thoracotomy with exposure of the ascending aorta 3 miniature PLGA films (1x1.5) carrying the total of 5μg LepA (superactive mouse leptin antagonist, a D23L/L39A/D40A/F41A mouse leptin mutant obtained from Protein Laboratories Rehovot, Ltd.)[1] were applied to the proximal ascending aorta, at the same location like leptin was applied in the leptin application model. Immediately after chest closure an osmotic minipump (Alzet, pump model 2004) containing AngII (human AngII, A9525 Sigma) was implanted in the interscapular subcutaneous space for AngII

infusion of 1000ng/kg/min and mice were subsequently followed for 28 days. During that period body weight, SBP, and echo of cardiovascular parameters was assessed (see below).

Blood pressure measurements:

Systemic blood pressure (SBP) measurements were performed using the BP-2000 Series II Blood Pressure Analysis System (Visitech Systems). Measurements were taken at baseline prior to surgery, then weekly for one month, and bi-weekly during the second month (in the leptin application model). Mice were trained on the BP system three times prior to baseline measurements. Two measurements per mouse were taken at every round. Each BP assessment per mouse consisted of 40 total measurements, once a minute. The first 10 measurements were discarded. Outliers were also excluded using the Chauvenet's criterion.

Echocardiography:

Echocardiography of the ascending aorta and left ventricle were performed at baseline, weekly/bi weekly (leptin model) and before termination of the experiment, using the Vevo 2100 system (Visualsonics). In each mouse, long axis and short axis B-mode views and videos were generated. Peak systolic velocity (PSV) was measured using Doppler mode at the LV outlet (aortic valve level). Accompanying software was used to measure aortic diameter and PSV. LV wall width and fraction area change (FAC) were extracted from videos generated by Vevo 2100 using custom MATLAB software. Experimental groups were blinded to the imager and analysts.

Mice tissues' analysis:

Mice were sacrificed with overdose anesthetic. The chest was opened to allow exposure of the heart and ascending aorta, followed by immediate perfusion fixation of the vascular tissues. A 10% formalin solution was infused through a LV puncture, flushing the heart and proximal aorta. The vascular organs, including the heart and full length aorta, were collected and exposed to further 8 hour fixation in 10% formalin, before being mounted in paraffin blocks.

Tissue 5μm sections on glass slides were deparaffinized and subjected to H&E staining or antibody retrieval in citric acid buffer (pH 6). Sections were then blocked with 0.3% peroxide and followed by normal horse serum. The following primary antibodies were used: mαhSMCactin (#M051, Dako) 1:150; RbαTgfβ (ab66043 Abcam) 1:50; RatαMac2 (#CL8942AP , Cedarlane) 1:100; Mac3 (#553322, BD Pharmingen) 1:100; RbαLeptin (ab16227, Abcam) 1:100; RbαKi67 (Dako), and were incubated overnight at 4°C. The Sigma #HT25A-1KT kit was used for Elastic Van Gieson staining. Image J software was used for quantification; experimental groups were blinded to analysts.

Human tissue samples:

The use of human tissues was approved by the Institutional Review Board of Sheba Medical Center, Tel Hashomer, Israel, and tissues were collected with informed consent. Clinicaltrials.org identifier NCT00449306.

Surgical samples of ascending aortic aneurysm were collected (n=11) from patients with a variety of background diseases, including hypertension (10), hypercholesterolemia (5), diabetes mellitus (2), Marfan's syndrome (4), bicuspid aortic valve (2), and ankylosing spondylitis (1) (table S1). Four patients were operated urgently for type A dissection.

Samples of human stenotic aortic valves (n=11) were collected from patients undergoing aortic valve replacement surgery. Normal aortic valves (n=3) were obtained from explanted hearts of patients undergoing heart transplantation.

Human tissues' analysis:

Paraffin-embedded human tissues were sectioned at 4 to 6μm and stained with H&E and modified Movat's pentachrome. Parallel sections were exposed to antibodies specific for αSMA (1:400; Dako) and the macrophage marker CD68 (anti-CD68 antibody, Kp-1 clone, 1:400; Dako). For identification of leptin and its specific receptor (LepR), paraffin cross-sections were incubated overnight at 4°C with a rabbit polyclonal antibody against human leptin or human LepR (1:200 or 1:100, respectively; Santa Cruz Biotechnology Inc.). When necessary, the antigen was retrieved by steam heat using EDTA (pH 8.0) or citrate buffer (pH 6.0). Antibody binding was visualized with a polymer-based HRP substrate (EnVision™; Dako) using NovaRED chromogen (Vector Laboratories) and Gill's hematoxylin (Sigma-Aldrich,) as counterstain. Formalin-fixed paraffin sections from normal human adrenal gland and small bowel served as positive controls for leptin and LepR, respectively.

***In situ* mRNA hybridization:**

Leptin mRNA *in situ* hybridization (ISH) was performed as previously described.[2] Briefly, 5μm paraffin sections were digested with proteinase K, followed by overnight incubations with human leptin RNA sense and antisense probes that had been *in vitro* transcribed and labeled with digoxigenin (Roche). Signals were visualized by antidigoxigenin AP antibodies (Roche) and nitroblue tetrazolium (Dako).

qPCR:

Reactions were performed on cDNA using an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) and Universal PCR Master Mix (Applied Biosystems) according to the manufacturer (Applied Biosystems). TaqMan probes and primers for leptin (assay ID number: HS00174877) and LepR (assay ID number: HS00174497) were 'assay-on-demand' gene expression products (Applied Biosystems). Total RNA converted to cDNA from two normal human aortic valves were used to establish a baseline for each detected gene to which all stenotic aortic valves (AVSs) samples were compared to. Normalization was performed using the endogenous control gene *Abl1* with the fluorescent probe 5'-Fam-CTGGCCCAACGATGGCGA-BHQ-3'.

The primers to *Abl1* gene used were:

forward: 5'-GGAGATAACACTCTAAGCATAACTAAAGG-3'

reverse: 5'-GATGTAGTTGCTTGGGACCCA-3'

The results presented are fold changes based on the differences of normalized Ct values compared to control samples, assuming optimal primer efficiency ($2^{-\Delta\Delta Ct}$). Results were analyzed using SDS 2.3 (Applied Biosystems) and Excel (Microsoft Corp) software.

***In vitro* studies**:

Valve interstitial cells (VICs), cell culture:

Human VICs (generously provided by Kristyn S Masters) were grown in MEMα + 15% FCS + 1.5% PSN + 1% Glutamine + 2.5 μg/mL Amphotericin B [Complete medium]. Cultures were split 1 to 3 every week to 10 days or after reaching >80% confluence, the earlier of them. The cells grow as long spindles, transforming into star-shaped cells,

forming colonies. Thereby, 80% confluence is relative to colonies' dimension more than surface covered. Medium was changed after 5-7 days in culture.

Proliferation experiments:

Cells were seeded in Complete medium in 24W plates (15,000 to 20,000 cells/well) and grown for 3-5 days, when the medium was changed to 1.5% FCS containing media for 48h (starvation period). Proliferation assay was performed in quadruplicates in fresh starvation medium supplemented with the tested factor for 24h. The degree of proliferation was measured by the XTT based Cell Proliferation kit (Biological Industries, Beit Haemek, Israel). Cells grown in starvation media only were used as control and their absorbance was 0.268 ± 0.030 O.D. (considered as 100%).

Quantitative real-time PCR

VIC P6 cells were grown to 80% confluency and treated with increasing concentrations of AngII for 4h or 24h. RNA was prepared from each 60 mm dish and converted to cDNA. RQ PCR was performed using an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) and Universal PCR Master Mix (Applied Biosystems) according to the manufacturer's instructions. The TaqMan probes and primers for *leptin* (assay ID number: HS00174877) and *leptin receptor* (assay ID number: HS00174497) were 'assay-on-demand' gene expression products (Applied Biosystems). The 4h and 24h untreated samples served as the controls thus normalized to 1 in the analysis. The endogenous reference gene control was TBP (assay ID number: HS99999910).The results presented are fold changes based on the differences of normalized Ct values compared to control samples, assuming optimal primer efficiency (2^ΔΔCt). Results were analyzed using SDS 2.3 (Applied Biosystems) and Excel (Microsoft Corp) software.

Each point assayed in triplicates, standard deviations (SD) were calculated and 2 tail T test was performed to calculate the statistically significances (p values).

Effect of AngII on Leptin and Leptin receptor mRNA expression in hVICs cultures:

Human VICs were cultured in 60 mm dishes in Complete medium. Cultures at 75-80% confluence were exposed to 1, 2.5 and 10 nM Angiotensin II (Sigma-Aldrich, St. Louis, MO, USA) for 4 and 24 hours in the same serum-containing medium. At the end of the incubation cultures were washed twice with complete DPBS (Biological Ind.) and total RNA was extracted using EZ-RNA (Biological Ind.) and the obtained RNA was dissolved in DEPC-treated water (Biological Ind.).

Statistics:

Two-sided Mann Whitney test was used to assess differences between Control and Leptin treated animals, or AngII vs. AngII and LepA treated animals. To overcome baseline variability between physiological parameters of ApoE-/- animals, each echo-cardiograph measurement at the end of the experiment was compared to a baseline measurement prior to surgery. All data is shown as mean ± standard error, and typical number of samples was 10 for mouse sample, and 5 for in vitro samples, as detailed in the figure captions. Fisher's exact test was used to assess the effect of local LepA treatment on thoracic aortic aneurysm rupture and death on AngII treated mice. The Benferroni correction was used for correction of proliferation and mineralization results in in vitro studies. Student's t-test was used for analysis of qPCR and mineralization data. The radar plot (Figure 7) scales physiological parameters by standard deviations in the untreated group.

References:

1. Shpilman M, Niv-Spector L, Katz M, Varol C, Solomon G, Ayalon-Soffer M, Boder E, Halpern Z, Elinav E, Gertler A. Development and characterization of high affinity leptins and leptin antagonists. J Biol Chem. 2011;286:4429–4442.

2. Schneiderman J, Schaefer K, Kolodgie FD, Savion N, Kotev-Emeth S, Dardik R, Simon AJ, Halak M, Pariente C, Engelberg I, Konstantinides S, Virmani R. Leptin locally synthesized in carotid atherosclerotic plaques could be associated with lesion instability and cerebral emboli. J Am Heart Assoc. 2012;1:e001727 doi: 10.1161/JAHA.112.001727.

Supplementary Figures

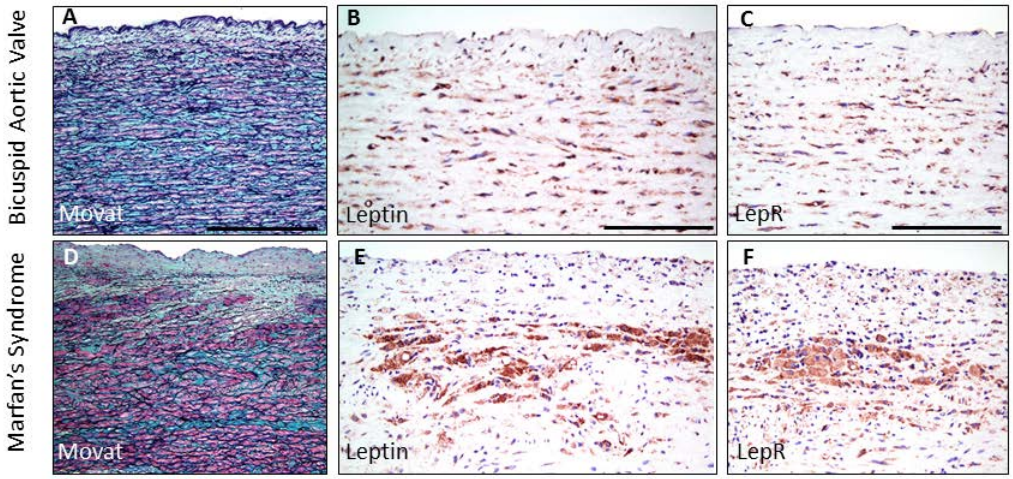

Figure S1: Movat pentachrome and immunohistochemistry for Leptin and LepR in ATAA samples from BAV and MFS patients; Subintimal and medial region.
A-C: BAV patient: Movat pentachrome (A), Diffuse elastic fiber fragmentation with glycosaminoglycan deposition (bluish-green) and media degeneration, Leptin (B), and LepR IHC (C), expressed by SMCs.
D-F: MFS patient: Movat pentachrome (D), histology similar to A, Leptin antigen (E) expressed mostly by medial macrophages, LepR antigen (F) prominently expressed in macrophages and SMCs.
A, D Scale bar=500μm; B, C, E, F scale bar=200μm.

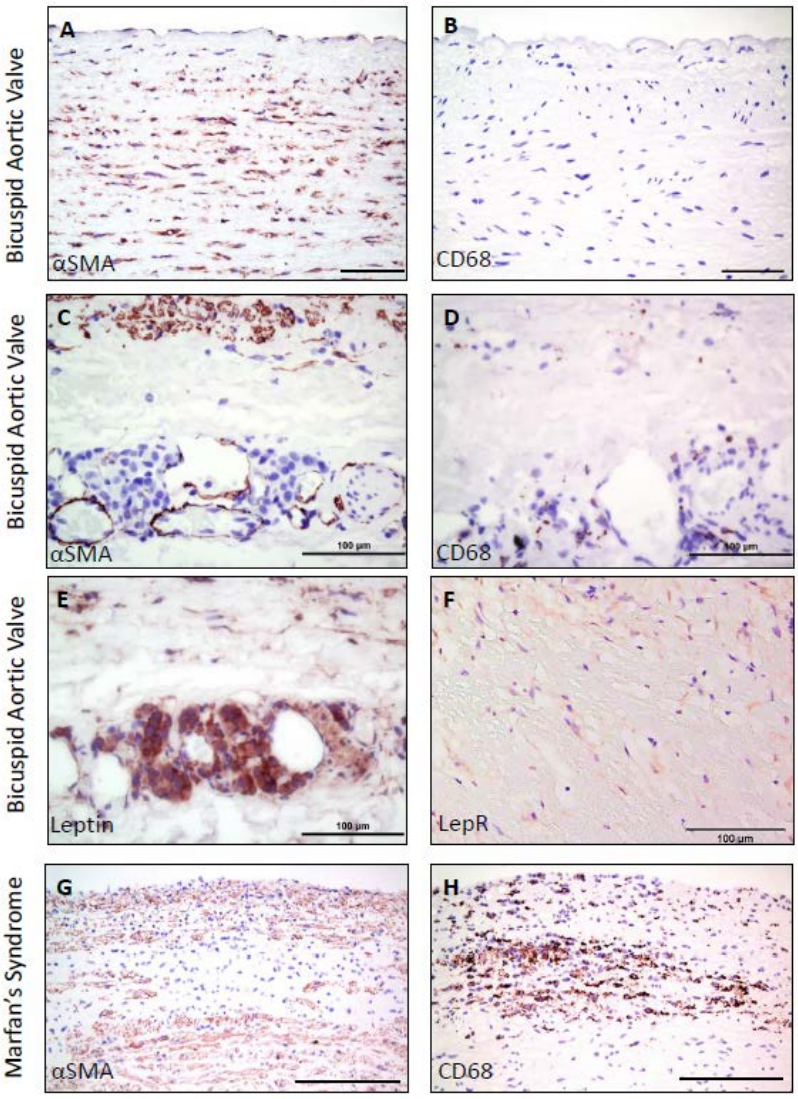

Figure S2: Immunohistochemical analysis of ATAA samples from BAV and MFS patients; Subintimal and medial region.
A-F analyses were performed on BAV related ATAA sample from a case shown in Suppl. Figure 1 A-C.
A, C: αSMA staining marking medial SMCs (A) and capillary SMCs (C),
B, D: CD68 marking macrophages was not identified in subintimal and medial region cells (B), however was evident in adventitial pericapillary cells (D).
E, F: Leptin antigen was expressed by pericapillary macrophages and surrounding adventitial SMCs (E), and LepR expressed mainly by medial SMCs (F).
A-F: Scale bar=100μm.
G,H Analysis performed on MFS related ATAA sample (same case, subintimal and medial region like in Suppl. Figure 1 D-F). αSMA staining marking smooth muscle cells (G) and CD68 marking macrophages (H). Scale bar=200μm.

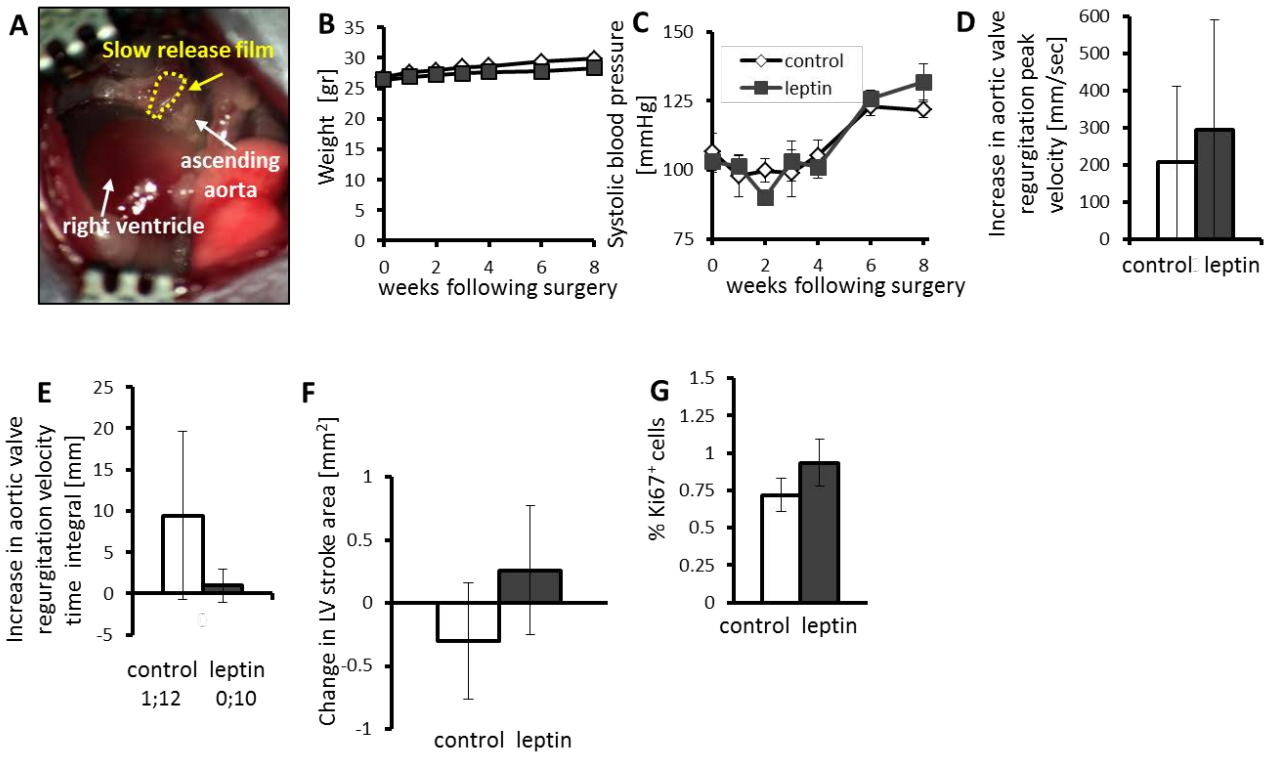

Figure S3: Effects of local leptin application on weight, systolic blood pressure and aortic valve proliferation.

A. An Intra-operative view showing the slow release PLGA film (yellow outline) placed adjacent to the ascending aorta. This strategy was used to apply the leptin slow release film, leptin antagonist, and control films as described in this study.

B. Weight of mice after receiving local leptin or control film. There was no significant difference in the weight between groups. N=10-11.

C. Systolic blood pressure of mice after receiving local leptin or control film. There was no significant difference in blood pressure between groups. N=10-11.

D. Increase in aortic valve regurgitation peak velocity following local treatment with leptin or control film. All animals displayed some regurgitation jet. Mean baseline velocity was 1000mm/sec.

E. Increase in aortic valve regurgitation velocity time integral (VTI). 1 out of 12 control animals displayed above baseline regurgitation, defined as VTI at least 10-fold greater than mean, 0 of 10 treated animals had regurgitation. Mean baseline velocity time integral was 2.8mm

F. Change in LV stroke area. N=10-11. Mean baseline stroke area was 5.9mm^2.

G. Percent of Ki67 positive stromal cells in aortic valve leaflets of mice receiving leptin or control film. Although there was a trend of increased Ki67 positive cells in leptin receivers vs controls, it did not reach statistical significance, N=4-5.

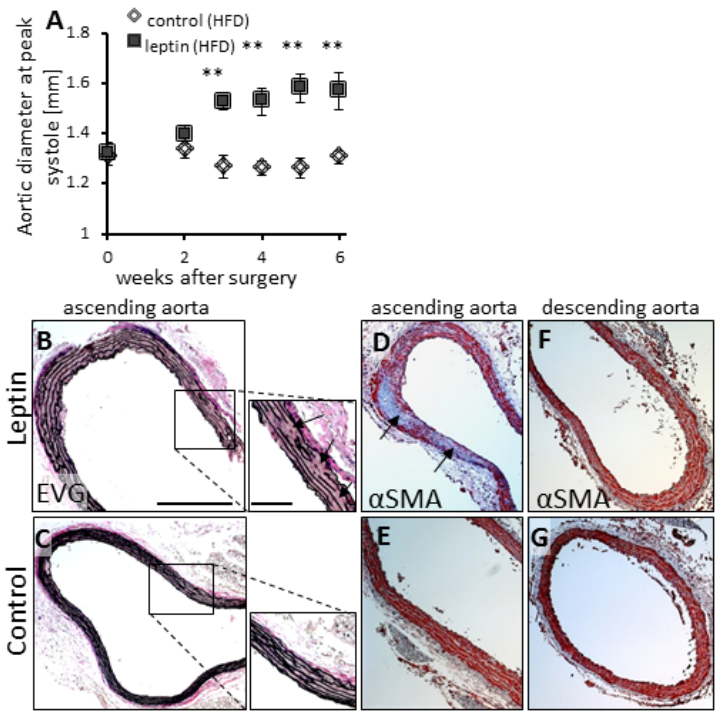

Figure S4: Modulation of the ascending aorta in mice receiving HFD feeding treated with control or leptin film.
A. Aortic diameter at peak systole in mice on HFD, treated with leptin or control film. After 3 weeks a significant difference was recorded between the two groups, $p<0.01$. n=5-6.
B-C. Elastic Van Gieson staining of the ascending aorta in local leptin (B) or control treated mice (C). Arrows in high magnification show fragmentation of elastic lamellas in leptin treated mice.
D-E. αSMA staining of the ascending aorta in local leptin (D) or control treated mice (E). Arrows indicate depletion of αSMA.
F-G. αSMA staining of the descending aorta in local leptin (F) or control mice (G). Leptin was applied to the ascending aorta. Note, medial SMC layer looks intact.
Scale bar=500μm for all images, and 100μm in high magnification images.
* $p<0.05$, ** $p<0.01$

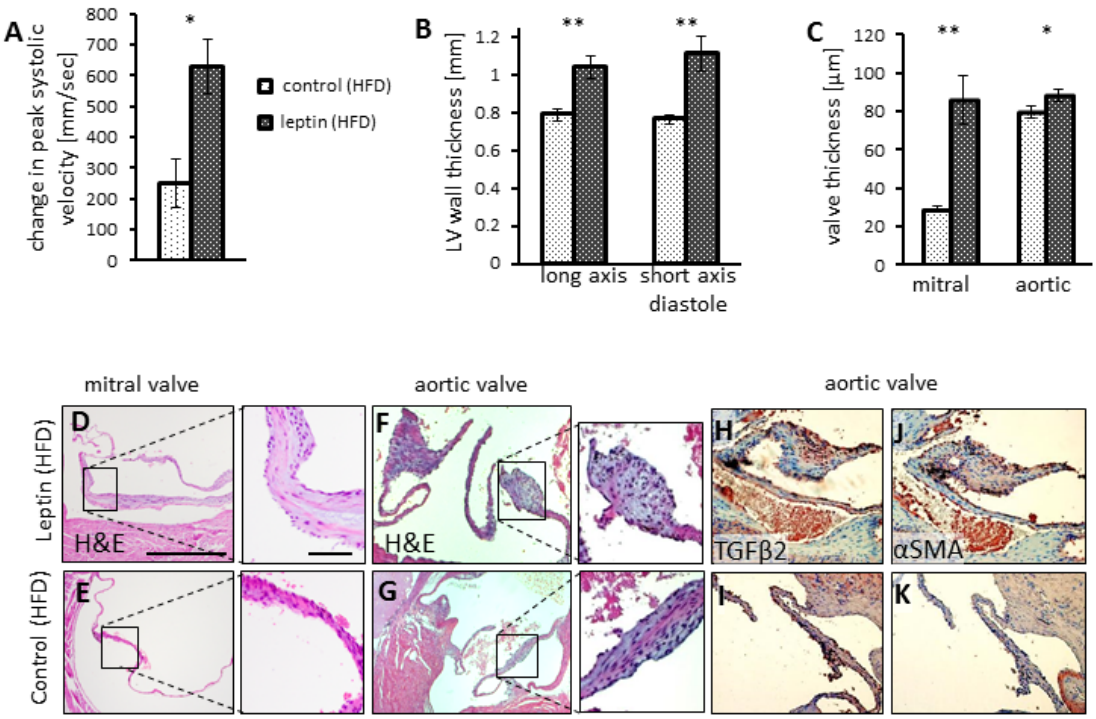

Figure S5: Cardiac and valvular modulation in mice on HFD, treated with local leptin or control film.

A. Change in PSV in control and leptin treated mice fed with HFD. p=0.04. Baseline PSV was 1300mm/sec.

B. LV wall thickness in leptin vs control treated HFD fed mice as measured by echocardiography 6 weeks after surgery in the long axis, and short axis view during diastole. $p<0.01$, n=5-6. Mean baseline LV wall thickness was 0.85mm.

C. Mitral and aortic valve thickness measured by histology 6 weeks after surgery. $p<0.01$ (mitral), p=0.047 (aortic).

D-E. H&E staining of mitral valve leaflets in local leptin (C) and control (E) treated mice.

F-G. H&E staining of aortic valve leaflets in local leptin (D) and control (F) treated mice.

H-I. TGFβ2 staining of aortic valve leaflets in local leptin (G) and control (H) treated mice. Positive signal indicates activation of stromal cells.

J-K. αSMA staining of aortic valve leaflets in local leptin (I) and control (J) treated mice.

Scale bar=500µm for all images, and 100µm in blow-ups.

* $p<0.05$, ** $p<0.01$

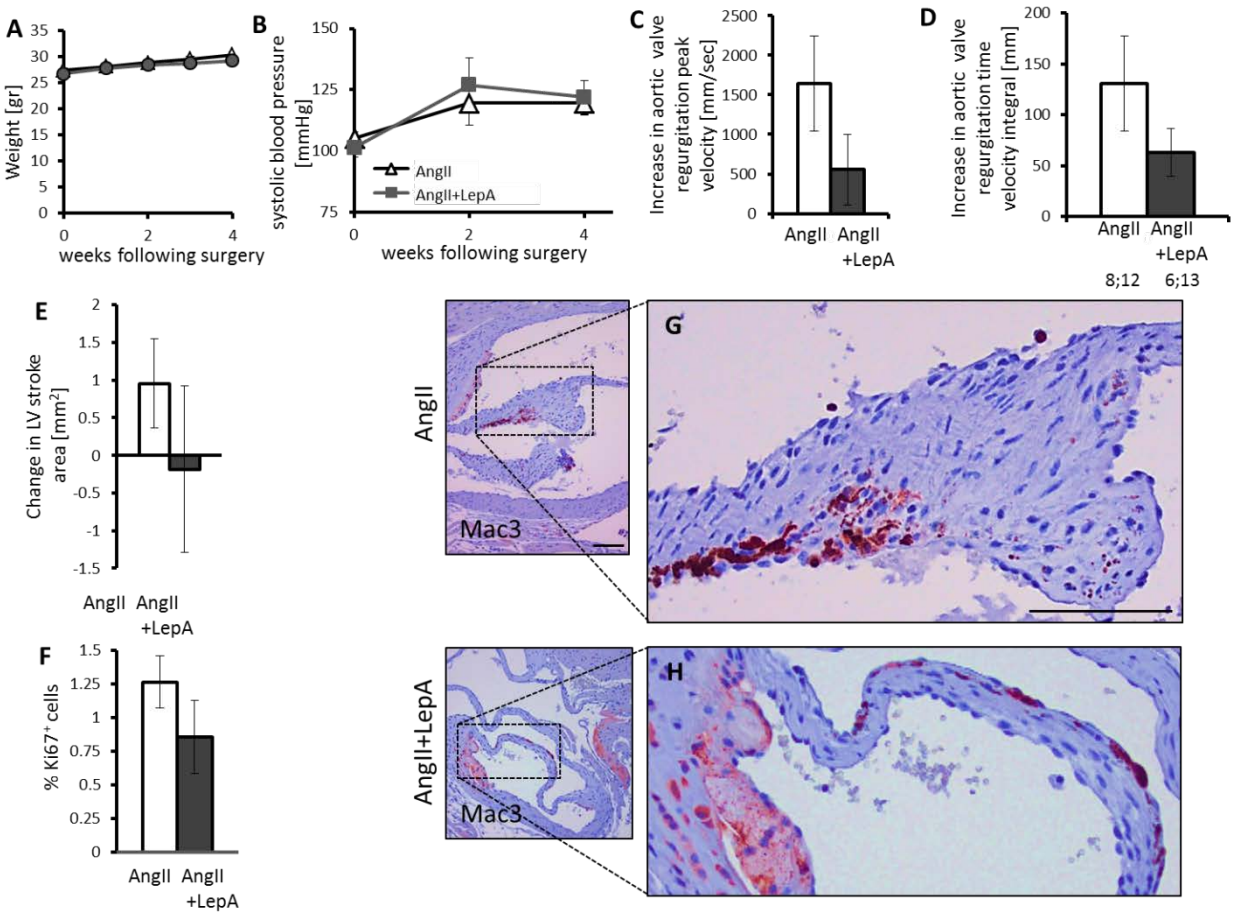

Figure S6: Effects of local application of LepA to the ascending aorta following AngII infusion

A. Weight of mice following treatment. There was no significant difference in weight between groups. N=11-12

B. Systolic blood pressure of mice after treatment. There was no significant difference in systolic blood pressure between groups. (N=11-12)

C. Increase in aortic valve regurgitation peak velocity following local treatment with leptin or control film. All animals displayed some regurgitation jet. Mean baseline peak velocity was 1050mm/sec

D. Increase in aortic valve regurgitation velocity time integral. 8 out of 12 AngII animals displayed aortic regurgitation, defined as at least 10-fold increase over baseline mean, 6 of 13 animals co treated with LepA had aortic regurgitation defined similarly. Mean baseline velocity time integral was 4.2mm.

E. Change in LV stroke area.N=11-12. Mean baseline stroke area was 6mm.

F. Percent of Ki67 positive cells in stromal cells in the aortic valve leaflets. N=4-5.

G-H. Mac3 staining of macrophages in aortic valve leaflets, in AngII (G) or AngII+LepA (H) treated mice. Scale bar=500μm.

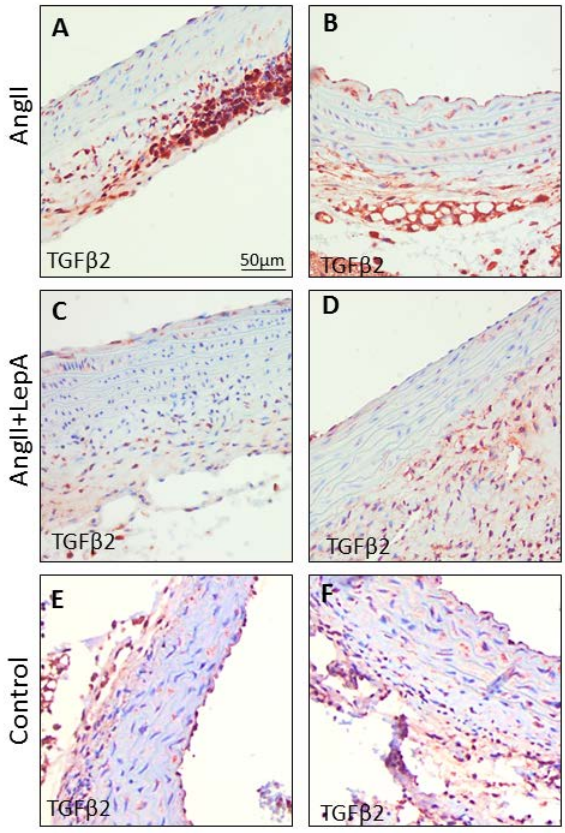

Figure S7: TGFβ2 localization in the ascending aorta following AngII infusion Immunostaining for TGFβ2 in ascending aortas of mice treated with AngII (A-B), AngII+LepA (C-D) or an empty film as control (E-F). Note, a weak to moderate TGFβ2 signal in medial SMCs, and strong expression in periadventitial macrophages in AngII receiving mice. When adding concomitant local LepA therapy, TGFβ2 antigen is absent in SMCs, and decreased in perivascular tissue, similar to controls. Scale bar=50μm.

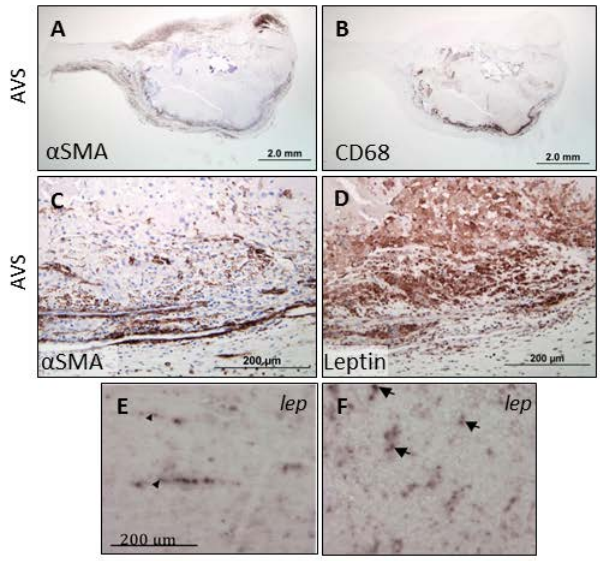

Figure S8: Immunohistochemical and *in situ* mRNA hybridization in stenotic aortic valves.
A-B. αSMA (A) and CD68 (B) staining in advanced AVS disease. Scale bar=2mm.
C-D. High magnification of αSMA (C) and Leptin (D) staining in advanced AVS. Scale bar=200µm.
E-F. *in situ* hybridization for *leptin* mRNA transcript in aortic valve leaflets from advanced AVS. Arrowheads (E) denote round macrophage like cells, arrows (F) denote elongated SMC like cells. Scale bar=200µm.