Supplemental Methods

Animal experiments

We studied osteogenic changes in arteries and aortic valves of 30-week-old apoE<sup>−/−</sup>/catS<sup>+/+</sup> (n=24) and apoE<sup>−/−</sup>/catS<sup>−/−</sup> (n=24) mice that consumed an atherogenic diet (Teklad TD 88137; 42% milk fat, 0.2% total cholesterol, Harlan, Indianapolis, IN) from 10 weeks of age. At 20 weeks of age, mice in each group were randomized either to continue with the atherogenic high-cholesterol diet or a CRD group (12 mice per group). Age-matched wild-type C57/BL6 mice (Jackson Laboratory, Bar Harbor, ME) with normal renal function (n=6) and CRD (n=6) served as controls. We used a two-step procedure to create CRD: left hemi-nephrectomy followed by right total nephrectomy one week later, procedures known to aggravate atherosclerosis.<sup>1,2</sup> At 10 weeks after surgery, mice underwent intravital microscopy and were sacrificed for ex vivo imaging and correlative histological analyses. The Subcommittee on Research Animal Care at Massachusetts General Hospital approved all procedures.

Blood biochemical analyses

Blood was collected from the heart into chilled dry tubes and spun in a refrigerated centrifuge, and serum was stored at -80°C. Serum levels of total cholesterol, cystatin C, creatinine, phosphate, and calcium were assessed (n=5 per group).

Creatinine assay

Mouse blood samples (n=5 per group) were spun for 10 minutes at 10,000 rpm and separated serum was again centrifuged for 60 min at 15,000 rpm to remove emulsionated fat. Cleared serum was assayed for creatinine levels using the standard
spectrophotometric Jaffe method according to manufacturer’s instructions (QuantiChrom Creatinine Assay kit, Bioassay Systems). Creatinine concentration was determined from absorption values at 490-530 nm.

**ELISA**

Serum samples from all groups of mice (n=5 per group) were collected and used for cystatin C ELISA. Briefly, 96-well immunosorb plates (NUNC Co., Naperville, IL) were coated with cystatin C polyclonal IgG (10 µg/mL; Cortex Biochem) in NaHCO$_3$ (pH 8.2) overnight at 4°C. After wash and 3% BSA blocking, plates were incubated with diluted mouse serum samples and were again incubated overnight at 4°C. Cystatin C mAb’s (1 µg/mL) were used as detecting antibody followed with peroxidase-conjugated goat anti-mouse IgG/IgM (Kirkegaard and Perry Laboratories, Gaithersburg, MD). ELISA plates were developed with OPD (Sigma Chemical Co.). Recombinant mouse cystatin C was used as standard for each plate.

**Molecular imaging agents**

**i) Detection of elastolytic activity.** A novel peptide-based cathepsin-S-activatable agent consists of backbone of a cathepsin-S-cleavable dipeptide substrate (Leu-Arg) and two polyethylene glycol (PEG) chains in their dendritic arms containing a fluorochrome.$^3$ Following enzymatic cleavage by cathepsin S, the fluorochromes separate, resulting in signal amplification (ex/em 750/780 nm). In vitro validation studies indicated that cathepsin S, but not cathepsins B, K, or L, cleave this probe.$^3$

**ii) Detection of osteogenic activity.** Bisphosphonate-conjugated imaging agent (OsteoSense680, VisEn Medical Inc., Woburn, MA) elaborates fluorescence evident
through the near-infrared window (ex/em 650/680 nm) detected osteogenic activity as previously described.\textsuperscript{4-6}

\textit{iii) Detection of inflammation.} Cross-linked iron oxide (CLIO-gly) fluorescent nanoparticle, an imaging agent that elaborates fluorescence visible through the near-infrared window (ex/em 750/780 nm) detected macrophage accumulation.

\textbf{Intravital laser scanning fluorescence imaging}

Mice (n=12 per group) simultaneously received two spectrally distinct imaging agents (activatable cathepsin S/750 and OsteoSense680 or CLIO750 and OsteoSence680) or saline via intravenous injection 24 hours before imaging. We performed dual-channel fluorescence imaging using an Olympus IV100 intravital laser scanning fluorescence microscope (Olympus Corporation, Tokyo, Japan) specifically developed for imaging small experimental animals as previously described.\textsuperscript{4, 5, 7} Images were acquired using an Olympus 4x UplanApo (N.A. 0.16) objective and the Olympus Fluoview FV300 program version 4.3. Samples were excited with a 633 nm HeNe-R laser (Model 05LHP925; Melles Griot) and a 748 nm infrared diode laser (Model FV10-LD748, Olympus Corporation, Tokyo, Japan) and images collected using custom build dichroic mirrors SDM-570 and SDM-750, and emission filters BA 650-700 nm and BA 770 nm IF respectively (Olympus Corporation, Tokyo, Japan). Images were acquired serially to avoid cross talk between channels and processed and analyzed using ImageJ software (v.1.41, NIH, Bethesda, MD).

\textbf{Macroscopic fluorescence reflectance imaging}

After mice were euthanized, aortas were perfused with saline, dissected, and imaged to map the macroscopic near-infrared osteogenic activity elaborated from
OsteoSense680 imaging agent using a fluorescence reflectance imaging system equipped with multichannel filter sets (Omega Optical, Brattleboro, VT) as previously described (n=12 per group).

**Histopathological assessment**

**i) Morphological characterization.** Tissue samples were frozen in OCT compound (Sakura Finetech, Torrance, CA) and 5-µm serial sections were cut and stained with hematoxylin and eosin for general morphology. Alkaline phosphatase activity (marker of early osteoblastic differentiation) was detected on cryosections according to the manufacturer’s instructions (Alkaline Phosphatase Substrate Kit; Vector Laboratories, Burlingame, CA). Van Gieson stain assessed elastin. Von Kossa silver stain histochemically imaged advanced calcification. Masson trichrome stain evaluated glomerulosclerosis.

**ii) Immunohistochemistry.** We employed immunohistochemistry for macrophages (anti-mouse Mac3, BD Biosciences, San Jose, CA) and cathepsin S (Santa Cruz Biotechnology, Santa Cruz, CA). Immunohistochemistry used avidin-biotin peroxidase method. The reaction was visualized with a 3-amino-9-ethyl-carbazol substrate (AEC, Sigma Chemical, Saint Louis, Missouri). Adjacent sections were treated with nonimmune IgG provided controls for antibody specificity. Images were captured with a digital camera (Nikon DXM 1200-F, Nikon Inc, Melville, NY). Fluorescence immunohistochemistry visualized simultaneously Texas Red-labeled cathepsin S and elastin. Images were captured and processed with the epifluorescence microscope (Eclipse 80i, Nikon Instruments, Melville, New York) with a cooled CCD camera (Cascade, Photometrics, AZ).
Real time RT-PCR

Total RNA was isolated from aorta by RNeasy Mini Kit (Qiagen) from all groups of mice (n=5 per group). Primer sequences were as follows: cathepsin-S, 5'-TCCCTCAGTGCTCAGACCT-3' and 5'-TGCTTTCTCAGGGCATCTT-3'; cathepsin-B, 5'-AAAAAGGCTGGTTTACAGGT-3' and 5'-GGGATAGCCACAGCTACAG-3'; cathepsin-K, 5'-AGACGCTTACCCGTATGTGG-3' and 5'-GGACACAGAGACGGTTCACT-3'; cathepsin-L, 5'-GTGACTTCTCAGCCTCA-3' and 5'-TTGACACTGCTGTCGACCT-3'; MMP 2, 5'-CCCCATGAACGCTTTGTTTACC-3' and 5'-TTGTAGGAGGTGCCGCTGA-3'; MMP 9, 5'-AGGTGGACCATGGTAAGTGAAC-3' and 5'-CGGTAGGAAGAGAAGGAG-3'. PCR detection and quantification of cathepsin S, B, L, and K used MyiQ Single-Color Real-Time PCR Detection System and iQ SYBR Green Supermix (BioRad). All measurements were duplicated and the mean value was used. Data are expressed in arbitrary units that were normalized by β-actin.

Primary human smooth muscle cell culture

Human vascular smooth muscle cells were plated on 12-well cell culture plates, maintained in DMEM containing 10% fetal bovine serum, allowed to reach 80%-90% confluence and then replaced with fresh calcification-promoting medium containing 0.9 mM NaH$_2$PO$_4$. Cells were treated with human aortic elastin peptides (molecular weight >1000), 100 mg/ml (Elastin Products Company), either alone or in the presence of human recombinant cathepsin S, 1 mg/ml (Calbiochem); cathepsin S plus the cathepsin inhibitor cystatin C, 1 mg/ml (Calbiochem); or cystatin C, 1 mg/ml. Separate plates were treated with 2 mM NaH$_2$PO$_4$ in addition to the above treatments. After 4 days of treatment, cells were washed twice with 100 mM TRIS pH 8.0 and stained for alkaline phosphatase activity. Cells were then fixed in 4% PFA for 15 minutes and re-stained for
the presence of calcium phosphate crystals using Von Kossa method. Stained cells were imaged with a color CCD camera (Nikon DXM 1200-F, Nikon Inc.), and the red stain indicative of alkaline phosphatase activity and the black silver staining indicative of calcium phosphate crystals were quantified as a percentage of total area using imaging software (IPLab version 3.9.3; Scalianic Inc., Rockville, MD).

**Quantification**

Macrophages were counted as a mean number of mac-3-positive cells per 10 high-power fields (x400) in the aortic root plaques or aortic valve lesions. Only cells expressing an antigen of interest (defined as red reaction product associated with blue hematoxylin nuclear counterstain) were counted as previously described.\(^8\)

**Statistical analysis**

Statistical analyses for comparison of multiple groups used one-way ANOVA followed by the Tukey post-hoc test was performed by using GraphPad Prism (v. 4.0, GraphPad Software, San Diego, CA). Student \(t\) test was performed when two groups were compared. Data are presented as mean ±SEM. \(P\)-values less than 0.05 were considered significant.
**Figure Legend**

**Histological evidence of kidney insufficiency in CRD mice.**

Histopathological analysis (Masson trichrome) reveals normal kidney appearance in apoE<sup>−/−</sup>catS<sup>+/+</sup> mice. CRD apoE<sup>−/−</sup>catS<sup>+/+</sup> and CRD apoE<sup>−/−</sup>catS<sup>−/−</sup> mice developed thickening of capsule membrane (arrows) and have enlarged glomeruli due to increased Bowman’s space, characteristic of renal insufficiency. Bar=50µm.
References


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