Supplemental Methods

Animal Protocol

We studied osteogenic changes in carotid arteries, aortic valves, and femur bones obtained from 30-week-old apoE-/- 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 were randomized either to continue the atherogenic diet (apoE-/-; n=10) or receive 5/6 nephrectomy procedure (CRD apoE-/-; n=10). We used 5/6 nephrectomy (left heminephrectomy, followed by right total nephrectomy one week later) to induce CRD, a procedure that also aggravates cardiovascular inflammation and calcification.[1-3] Age-matched wild-type C57/BL6 mice (WT, n=8, Jackson Laboratory, Bar Harbor, ME) served as controls. At 30 weeks of age, mice underwent intravital microscopy and were euthanized for *ex vivo* imaging following by statistical analyses. In addition to molecular imaging, the femurs of the mice underwent quantitative micro-CT imaging analyses (WT, n=3; apoE-/-, n=4; CRD apoE-/-, n=4). The Subcommittee on Research Animal Care at Massachusetts General Hospital approved all procedures.

Blood biochemical analyses

Blood was obtained from the heart, and serum levels of total cholesterol, cystatin C, creatinine and phosphate were assessed as previously described.[3]

Molecular imaging agents

To image osteogenesis, we used biphosphonate-conjugated imaging agent (Osteosense680/OS680, VisEn Medical, Inc., Woburn, MA) binding to hydroxyapatite in actively mineralized regions containing osteoblast-like cells in arteries, valves (**Suppl. Figure 1A**) [3-5] and bones.[6] This agent elaborates fluorescence detectable through the near-infrared (NIR) window excitation/emission 650/680nm. Macrophage-targeted near-

infrared (NIR)-conjugated iron nanoparticle (**Suppl. Figure 1B**), producing fluorescence visible through the NIR window excitation/emission 750/780nm, was used to detect inflammation.[3, 5]

Molecular imaging of carotid arteries, aortic valves, and femurs

Mice simultaneously received two spectrally distinct imaging agents (OsteoSense680 and CLIO-750) via intravenous injection 24 hours before imaging. Dual-channel fluorescence imaging was performed using an intravital laser scanning fluorescence microscope specifically developed for imaging of small experimental animals. Excitation at 633 and 748 nm and image collection in two different channels was done serially to avoid cross talk between channels. Image stacks were processed and analyzed with ImageJ software (version 1.41, Bethesda, MD). Image post-processing was done with OsiriX imaging software (version 3.6.1, Open Source). Data was presented as region of interest (ROI; pixels) and signal intensity (SI; Arbitrary Units; AU).

Macroscopic fluorescence reflectance imaging of aortic specimens

After intravital imaging, mice were euthanized. Aortas were perfused with saline, dissected, and imaged to map the macroscopic NIR fluorescent signals elaborated from Osteosense680 using fluorescence reflectance imaging system (Omega Optical, Brattleboro, VT).[3, 4]

Micro-CT imaging of bones

Whole femurs were transferred to individual conical tubes filled with fresh PBS and imaged using micro-CT (GE eXplore Vision CT 120, GE Healthcare, Inc.). Bones were scanned at 90 KeV and 40mA, using 1200 angles and 4 images per angle to enhance clarity. Calibrations and comparison with scans by other micro-CT systems

demonstrated that this protocol results in 25 µm voxel size. A calibrated bone phantom (SB3 with 1073 mg/cc, GE Healthcare, Inc.) was scanned simultaneously with the experimental samples to relate X-ray attenuation by different scans. Image stacks were reconstructed to 3D datasets using the eXplore software and transferred to MicroView v3.2 (GE Healthcare, Inc.) for post processing and analysis. The midshaft cortical region and the trabecular region in the femoral head were analyzed. For the midshaft, a 70-slice segment representing 1.75 mm axial length was processed using MicroView as previously described. Bone was identified in each slice via X-ray attenuation–based Hounsfeld unit thresholding. The average cortical and marrow areas for each bone were then determined. The 3D cortical bone volume was segmented out of the original dataset and tissue mineral density was calculated using MicroView. For the trabecular analysis, a trapezoidal zone between the epiphysis and the metaphysis containing only trabecular networks was extracted and analyzed using MicroView. Bone volume fraction, trabecular number, thickness, and spacing were quantified for each treatment. At least three femurs were analyzed for each experimental condition.

Correlative histopathological assessment

After imaging, tissue samples were frozen in OCT compound (Sakura Finetech, Torrance, CA), and 5 um sections were cut through the carotid arteries and aortic valves. Alkaline phosphatase activity was detected on cryosections that were directly incubated with conjugated antibody (Vector Labs, Burlingame, CA). The avidin-biotin peroxidase method was used for immunohistochemistry for detection of macrophages (rat monoclonal antibody against mouse Mac 3, BD Biosciences, San Jose, CA). The reaction was visualized with a 3-amino-9-ethylcarbazol substrate (AEC, Sigma Chemical, St Louis, MO). Images were captured with a digital camera (Nikon DXM 1200-F, Nikon Inc, Melville, NY).

Statistical analyses

Data are presented as mean +/- standard deviation. Statistical analyses for comparison groups employed One-way ANOVA, followed by the Tukey-Kramer post hoc test performed with GraphPad prism software (version 4.0, GraphPad Software, San Diego, CA). Tests were two-sided and adjusted for variance inequality where appropriate. Linear regression with Pearson's correlation test was used to determine covariant relationships between data. Normal distribution of the data was confirmed by comparing against expected normal distributions with given means and standard deviation. In all cases, test probability values less than 0.05 were considered statistically significant. For intravital imaging, signal intensity (SI) and region of interest (ROI) were identified as positive area detected by OsteoSense680- or CLIO750-derived signals and selected in each sum-image using ImageJ imaging software (v. 1.41, Bethesda, MD) as previously described.[4]

References:

1. Massy ZA, Ivanovski O, Nguyen-Khoa T, Angulo J, Szumilak D, Mothu N, Phan O, Daudon M, Lacour B, Drueke TB, Muntzel MS. Uremia accelerates both atherosclerosis and arterial calcification in apolipoprotein E knockout mice. *J Am Soc Nephrol.* 2005;16(1):109-116.

2. Bro S, Bentzon JF, Falk E, Andersen CB, Olgaard K, Nielsen LB. Chronic renal failure accelerates atherogenesis in apolipoprotein E-deficient mice. *J Am Soc Nephrol.* 2003;14(10):2466-2474.

3. Aikawa E, Aikawa M, Libby P, Figueiredo JL, Rusanescu G, Iwamoto Y, Fukuda D, Kohler RH, Shi GP, Jaffer FA, Weissleder R. Arterial and aortic valve calcification abolished by elastolytic cathepsin S deficiency in chronic renal disease. *Circulation.* 2009;119(13):1785-1794.

4. Aikawa E, Nahrendorf M, Figueiredo JL, Swirski FK, Shtatland T, Kohler RH, Jaffer FA, Aikawa M, Weissleder R. Osteogenesis associates with inflammation in early-stage atherosclerosis evaluated by molecular imaging in vivo. *Circulation.* 2007;116(24):2841-2850.

5. Aikawa E, Nahrendorf M, Sosnovik D, Lok VM, Jaffer FA, Aikawa M, Weissleder R. Multimodality molecular imaging identifies proteolytic and osteogenic activities in early aortic valve disease. *Circulation.* 2007;115(3):377-386.

6. Kozloff KM, Volakis LI, Marini JC, Caird MS. Near-infrared fluorescent probe traces bisphosphonate delivery and retention in vivo. *J Bone Miner Res.*