Supplemental material

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Inhibition of bone morphogenetic protein signaling reduces vascular calcification and atherosclerosis

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Supplemental Tables

Supplementary Table I

	NCBI Gene ID	Sequence
BMP2 forward	650	ACCCGCTGTCTTCTAGCGT
BMP2 reverse		CTCAGGACCTCGTCAGAGGG
BMP4 forward	652	TTCCTGGTAACCGAATGCTGA
BMP4 reverse		CCCTGAATCTCGGCGACTTTT
BMP6 forward	654	AGCGACACCACAAAGAGTTCA
BMP6 reverse		GCTGATGCTCCTGTAAGACTTGA
BMP7 forward	655	CGCCGCCTACTACTGTGAG
BMP7 reverse		AGGTGACCACACCCCAAGAT
BMP9 forward	2658	AGAACGTGAAGGTGGATTTCC
BMP9 reverse		CGCACAATGTTGGACGCTG

Supplementary Table I. List of primers used for quantitative RT-PCR.

Supplementary Table II

	Vehicle	LDN-193189	p=
Cholesterol [mg/dl]	1957 ± 159	1401 ± 87	0.01
Triglycerides [mg/dl]	125 ± 23	135 ± 16	0.73
Hemoglobin [g/dl]	11.6 ± 1.0	12.9 ± 0.8	0.34
Blood urea nitrogen [mg/dl]	25 ± 1	25 ± 2	0.84
Glucose [mg/dl]	216 ± 21	230 ± 19	0.65
Alkaline phosphatase [IU/L]	158 ± 15	84 ± 11	0.00
Total protein [g/dl]	4.9 ± 0.1	4.3 ± 0.4	0.12
Alanine transaminase [IU/L]	257 ± 44	130 ± 21	0.03
Creatinine [mg/dl]	0.5 ± 0.0	0.4 ± 0.0	0.61
	n=10	**n=8**	

Supplementary Table II. Blood biochemical analysis in LDLR$^{-/-}$ mice fed a high fat diet. LDLR$^{-/-}$ mice were started on a HFD at eight weeks of age that was continued for 20 weeks during which mice received daily injections of either vehicle or LDN-193189 (2.5 mg/kg ip, data are presented as mean ± SEM).

Supplementary Table III

	Vehicle	LDN-193189	p=
Cholesterol [mg/dl]	218 ± 5	183 ± 6	0.00
Triglycerides [mg/dl]	144 ± 26	82 ± 21	0.10
Hemoglobin [g/dl]	13.7 ± 0.9	13.5 ± 1.0	0.89
Blood urea nitrogen [mg/dl]	32 ± 4	25 ± 1	0.09
Glucose [mg/dl]	283 ± 16	311 ± 12	0.17
Alkaline phosphatase [IU/L]	177 ± 12	109 ± 8	0.00
Total protein [g/dl]	4.8 ± 0.1	4.7 ± 0.2	0.56
Alanine transaminase [IU/L]	275 ± 21	159 ± 19	0.00
Creatinine [mg/dl]	0.6 ± 0.0	0.5 ± 0.0	0.06
	n=8	**n=9**	

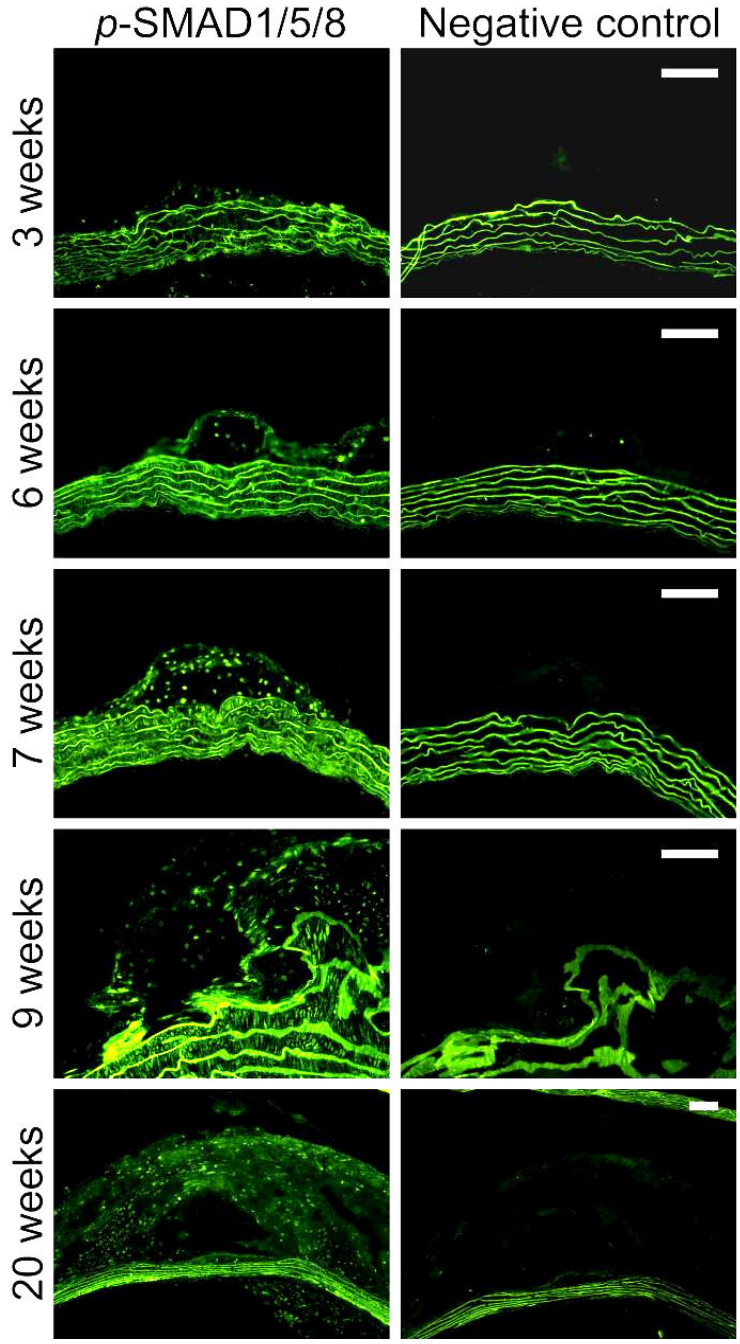
Supplementary Table III. Blood biochemical analysis in WT mice fed a HFD for 30 weeks. C57BL/6 mice were started on a HFD at eight weeks of age that was continued for 30 weeks during which mice received daily injections of either vehicle or LDN-193189 (2.5 mg/kg ip, data are presented as mean ± SEM).

Supplementary Table IV

	Vehicle	ALK3-Fc	LDN-193189
Cholesterol [mg/dl]	1953 ± 102	2206 ± 139	1553 ± 77*,§
Triglycerides [mg/dl]	122 ± 10	108 ± 11	112 ± 15
Hemoglobin [g/dl]	13.7 ± 1.3	14.2 ± 1.1	12.9 ± 1.5
Blood urea nitrogen [mg/dl]	28 ± 1	25 ± 1	24 ± 2
Glucose [mg/dl]	253 ± 19	243 ± 16	259 ± 20
Alkaline phosphatase [IU/L]	141 ± 14	207 ± 22	112 ± 20§
Total protein [g/dl]	4.9 ± 0.1	5.2 ± 0.1	5.1 ± 0.1
Alanine transaminase [IU/L]	408 ± 74	310 ± 72	233 ± 56
Creatinine [mg/dl]	0.5 ± 0.1	0.6 ± 0.0	0.5 ± 0.1
	n=9	**n=9**	**n=9**

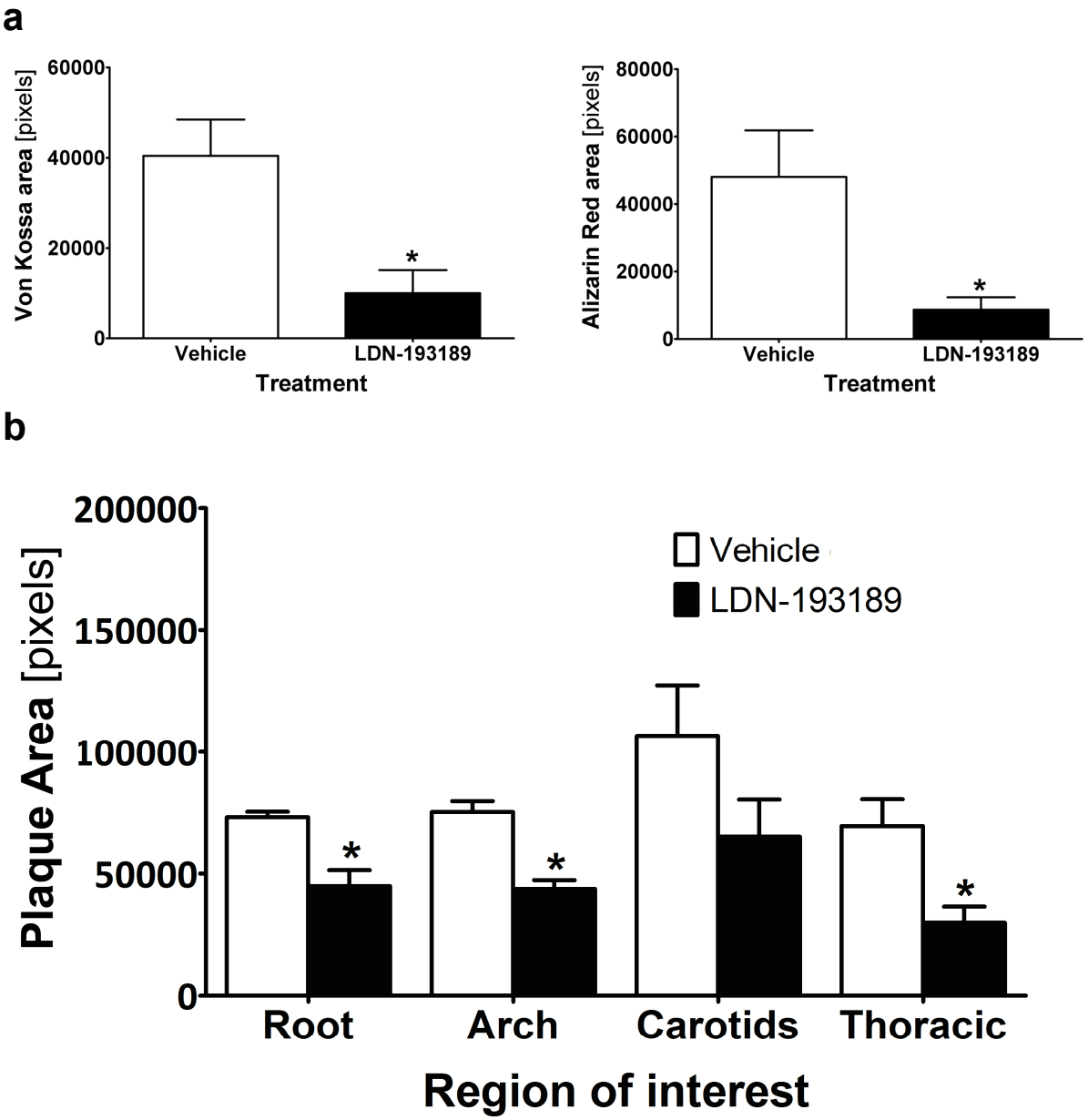
Supplementary Table IV. Blood biochemical analysis in LDLR$^{-/-}$ mice fed a high fat diet. LDLR$^{-/-}$ mice were started on a HFD at eight weeks of age that was continued for 6 weeks during which mice received daily injections of vehicle or LDN-193189 (2.5 mg/kg ip) or received ALK3-Fc (2 mg/kg ip) every other day. Data are presented as mean±SEM. *p≤0.05 LDN-193189 vs. vehicle. §p≤0.05 LDN-193189 vs. ALK3-Fc.

Supplementary Figure I



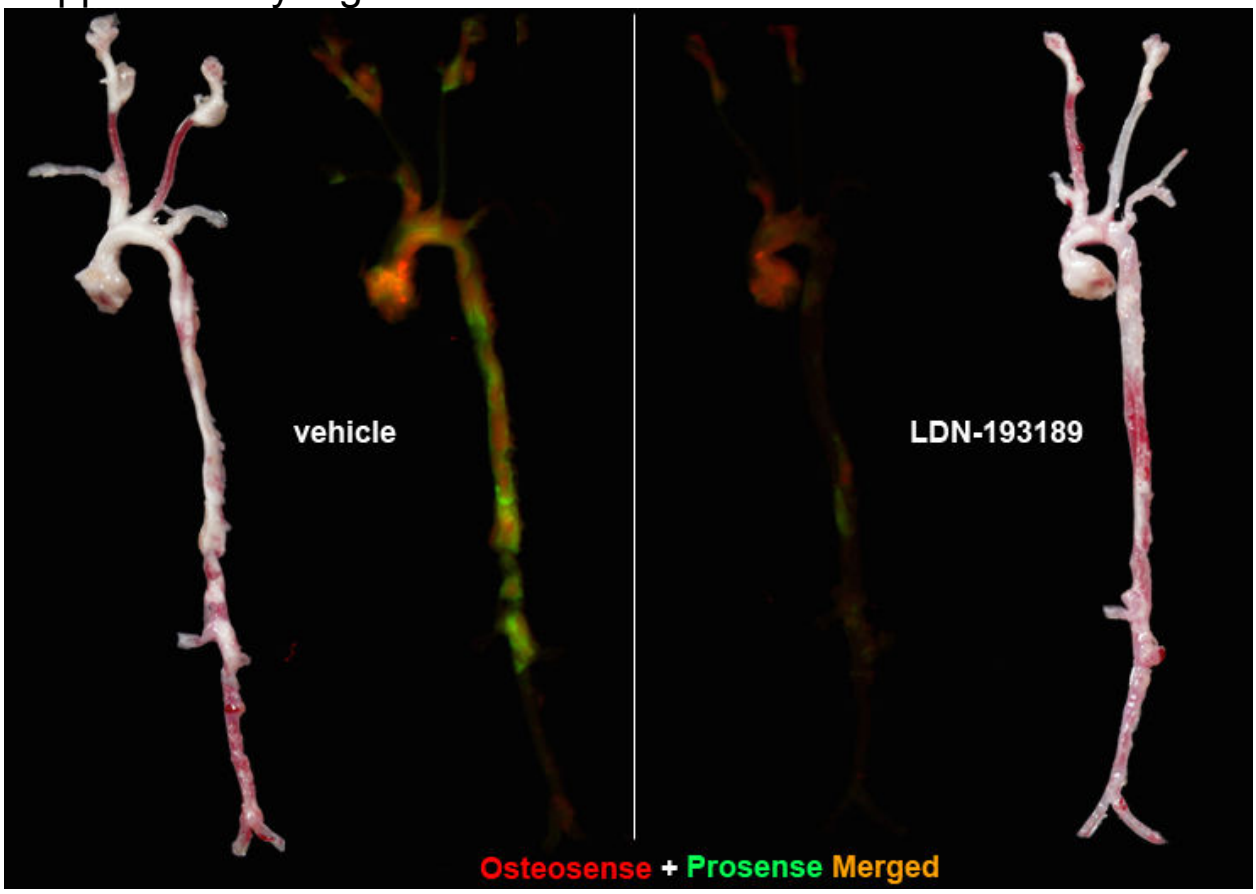
Supplementary Figure I. LDLR-/- mice on HFD develop atherosclerotic lesions demonstrating marked activation of SMAD1/5/8. Aortic sections from LDLR$^{-/-}$ mice fed HFD for 3, 6, 7, 9, and 20 weeks showed evidence evidence of phospho-(*p-)*SMAD immunoreactivity in evolving atheromatous plaques (left panels), as compared to serial sections from the same aortae reacted with FITC-labeled secondary Ab alone (right panels). Nuclear staining for *p*-SMAD1/5/8 was observed in the intimal, subintimal, and medial areas of involvement in atheromatous lesions (images representative of ≥3 aortae at each interval, bar= 500 μm).

Supplementary Figure II



Supplementary Figure II. Reduction of vascular calcification and atheroma formation in conventional histology. (**a**) Calcified surface area (**left panel**) determined by von Kossa-staining of longitudinal sections from the aortic minor curvature from LDLR$^{-/-}$ mice fed HFD for 20 weeks was markedly reduced with LDN-193189 treatment (n=5, 2.5 mg/kg ip) vs. vehicle (n=5, $p<0.05$). Similarly, Alizarin Red staining of serial sections from the same aortae revealed decreased areas calcification in LDN-193189-treated vs. vehicle-treated mice (n=4 each group, $p<0.05$) (**b**) Lipid plaque surface area, determined by areas of Oil Red O staining in whole-mount aortae from LDLR$^{-/-}$ mice fed HFD for 20 weeks, was significantly reduced in animals receiving LDN-193189 (n=3, 2.5 mg/kg ip) vs. vehicle (n=3, mean±SEM, *p <0.05).

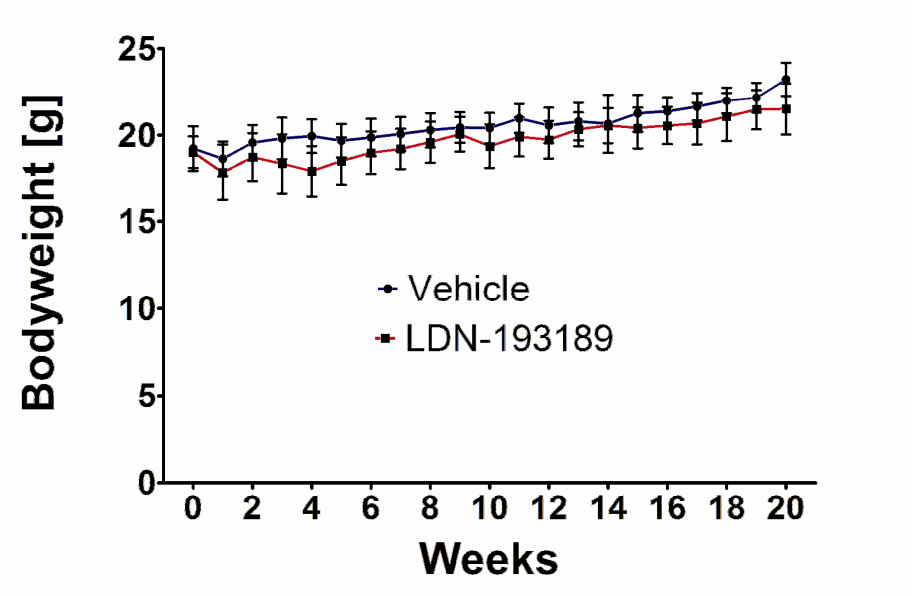
Supplementary Figure III



Supplementary Fig. III. Vascular calcification and inflammation in aortae from LDLR$^{-/-}$ mice are detected by ex vivo molecular imaging with Osteosense and Prosense in overlapping but distinct areas of the aorta, and are both inhibited by treatment with a BMP antagonist. Aortae were harvested from HFD-fed LDLR$^{-/-}$ mice treated with vehicle (**left panel**) or LDN-193189 (**right panel**) for 20 weeks, dissected and imaged by near-infrared fluorescence reflectance imaging 24h after iv injection with OsteoSense 680 (a near-infrared fluorescent bisphosphonate probe). Brightfield images (outside) correspond to colorized intensity maps (inside) demonstrating localization and degree of osteogenic activity (red) and inflammation (green). Treatment with LDN-193189 diminished aortic osteogenic activity, and markedly diminished vascular inflammation.

Supplementary Figure IV

a



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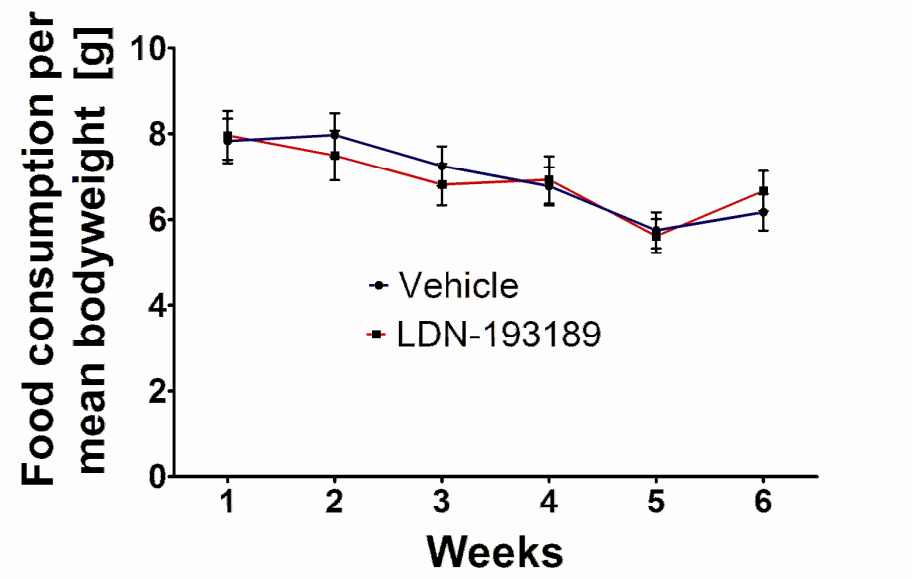
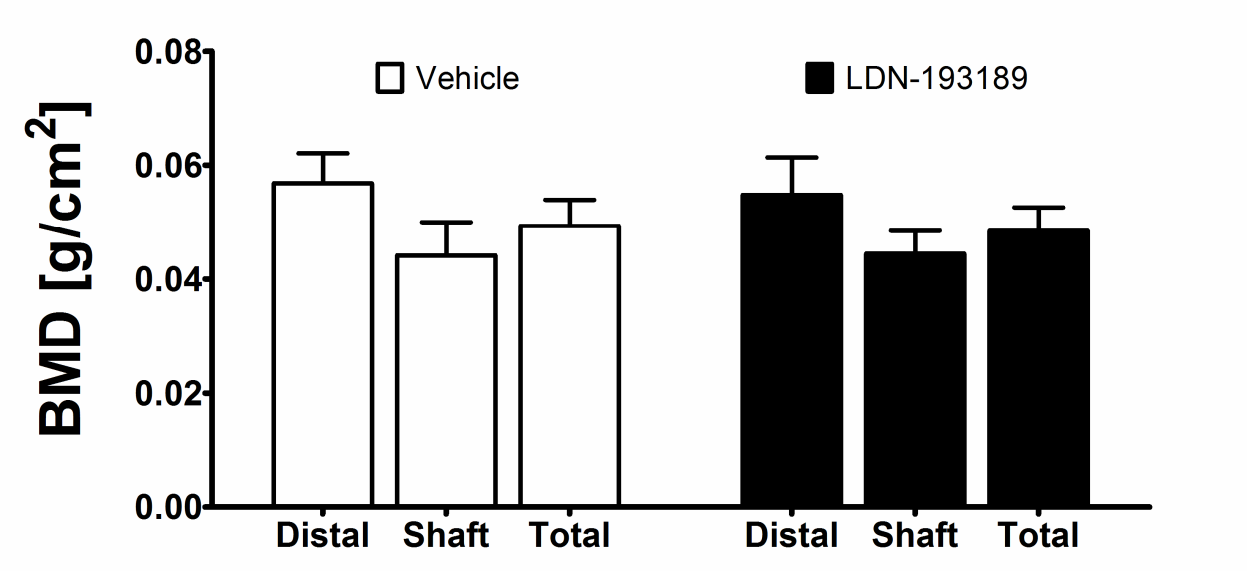


Supplementary Figure IV. Body weight and food intake did not differ between LDLR$^{-/-}$ mice treated with vehicle or LDN-193189. (**a**) Mean weight (g) in LDLR$^{-/-}$ mice fed a HFD for 20 weeks while receiving daily injections of vehicle (n=20) or LDN-193189 (n=20, 2.5 mg/kg ip). (**b**) Food intake per g weight over 6 weeks of HFD administration while receiving daily injections of vehicle (n=10) or LDN-193189 (n=10, 2.5 mg/kg ip). Data are presented as mean ± SEM.

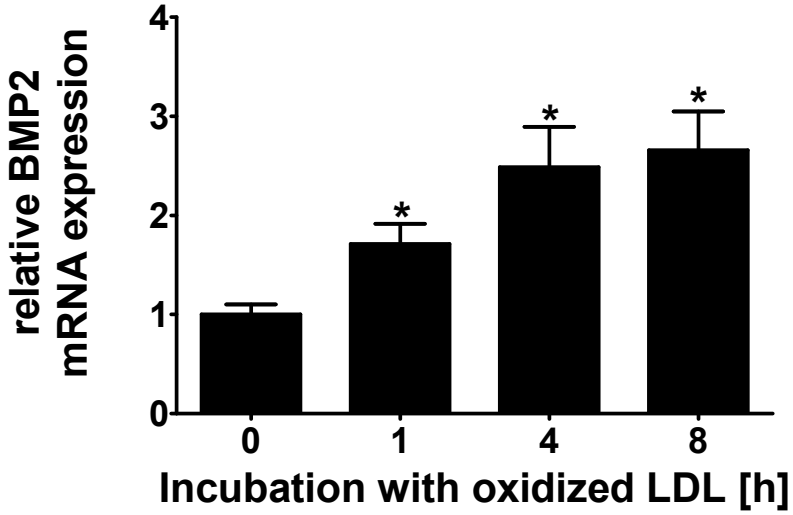
Supplementary Figure V



Supplementary Figure V. Bone mineral density did not differ between LDLR$^{-/-}$ mice treated with vehicle or LDN-193189. Bone mineral density (BMD) was measured in femurs from sacrificed LDLR$^{-/-}$ mice fed a HFD for 20 weeks while receiving daily injections of vehicle (n=8) or LDN-193189 (n=10, 2.5 mg/kg ip) using dual energy X-ray absorptiometry in the distal femur (Distal), the femur shaft (Shaft) or in the whole bone (Total, mean ± SEM).

Supplementary Figure VI

a



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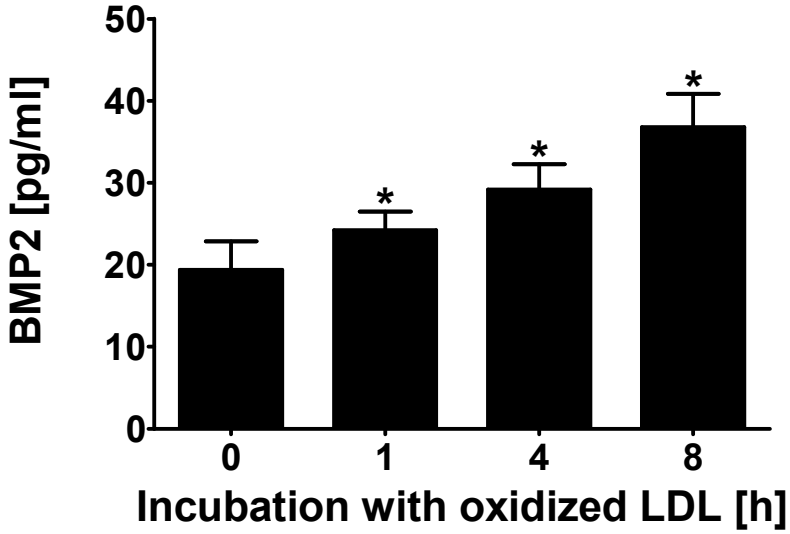
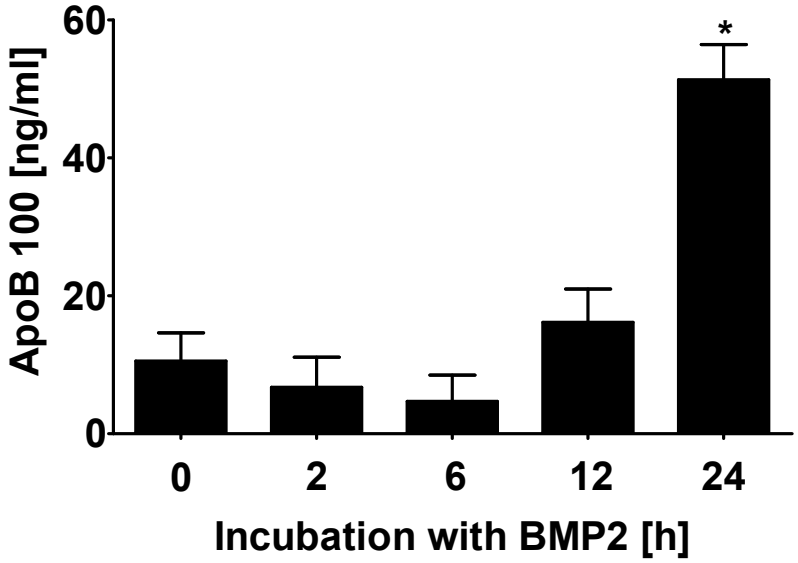


Supplementary Figure VI. BMP2 is induced in human aortic endothelial cells by oxidized LDL. (**a**) BMP2 mRNA levels were measured by quantitative RT-PCR. Data presented as mean±SEM, n=4 measurements. (**b**) BMP2 protein levels in the culture medium were measured using the BMP-2 Quantikine ELISA Kit (DBP200, R&D Systems, Minneapolis, MN). BMP2 mRNA and protein levels increased over time in response to incubation with oxLDL (80 µg/mL). Data presented as mean ± SEM, n=4 measurements. *p≤0.05 versus HAEC not exposed to oxLDL (0 h).

Supplementary Figure VII

a



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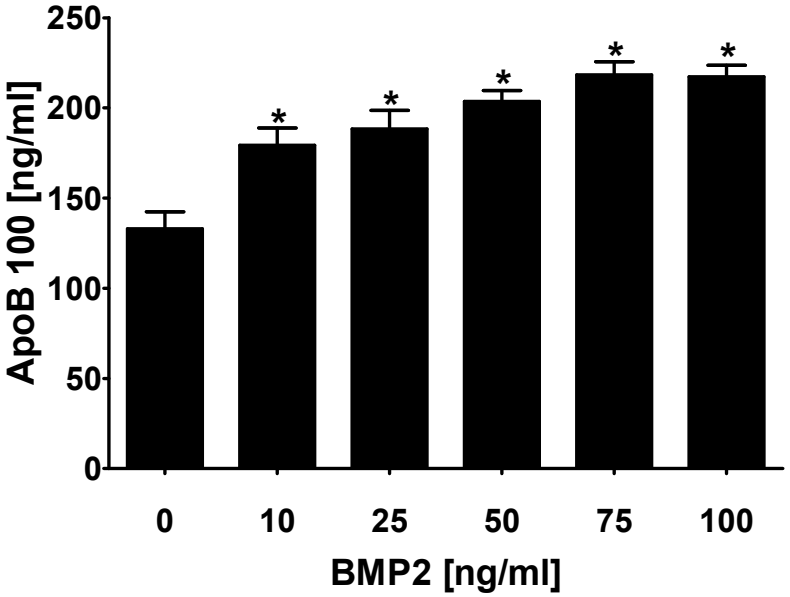
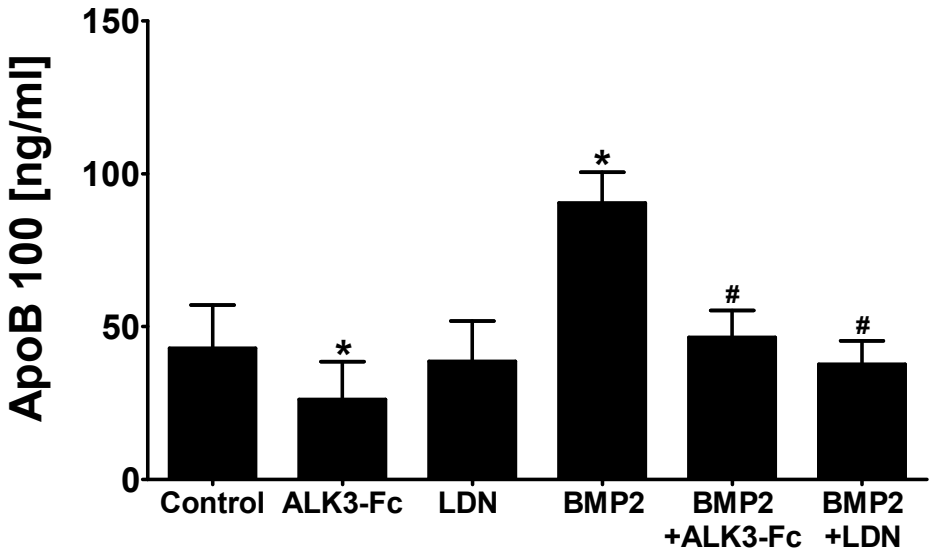


Supplementary Figure VII. BMP2 induces Apolipoprotein B 100 production in a time- and dose- dependent manner in HepG2 cells. (**a**) After starvation in EMEM culture media containing 0.1% fetal bovine serum for 24 h, HepG2 cells were incubated with BMP2 (100 ng/mL) for varying periods of time. Apolipoprotein B 100 (ApoB) levels, measured in culture medium by ELISA, were increased after 24 h of BMP2 stimulation (mean ± SEM, n=4, *p ≤ 0.05 vs. control). (**b**) After starvation in EMEM culture media containing 0.1% fetal bovine serum for 24 h, cells were incubated with varying concentrations of BMP2 for 24 h. Apo B levels increased with BMP2 stimulation in a dose-dependent fashion (mean ± SEM, n=4, *p≤0.05 vs. control, Pearson's correlation, $p<0.001$).

Supplementary Figure VIII



Supplementary Fig. VIII. Recombinant or small molecule inhibition of BMP signaling inhibits BMP2-induced ApoB production. After serum deprivation for 24 h, HepG2 cells stimulated with BMP2 (100 ng/mL) for 24 h secreted ApoB 100 into secretion in a manner that was inhibited by ALK3-Fc (400 ng/mL) or LDN-193189 (LDN, 100 nM, mean ± SEM, n=4, *p≤0.05 vs. control, $^{\#}$p≤0.05 vs, BMP2).