

Supplemental Material

Gut microbial metabolite imidazole propionate impairs endothelial cell function and promotes the development of atherosclerosis

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Supplementary Figures:

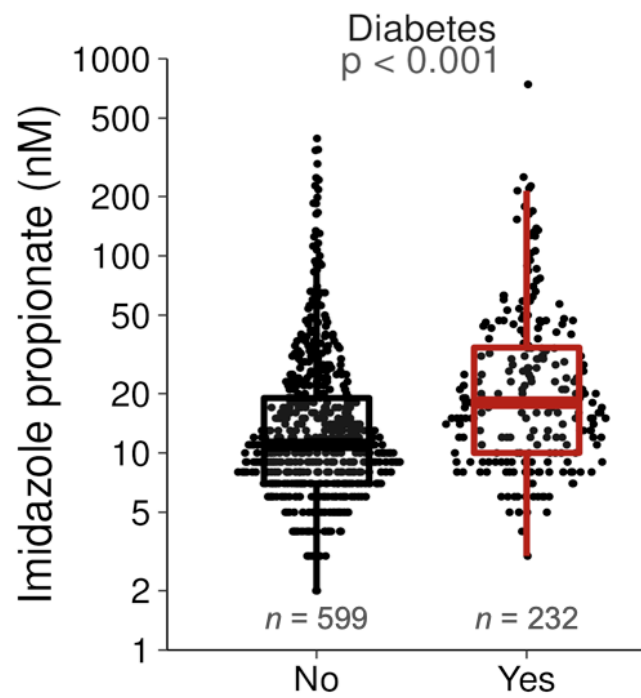


Figure S1: ImP is increased in patients with type 2 diabetes. Graph shows the comparison of ImP levels between patients without and with type 2 diabetes ($n = 831$). Data is shown as boxplots demonstrating the middle line as the median, the lower and upper hinges as the first and third quartiles, and the whiskers represent 10th and 90th percentiles; P -values were analyzed using Wilcoxon rank sum test.

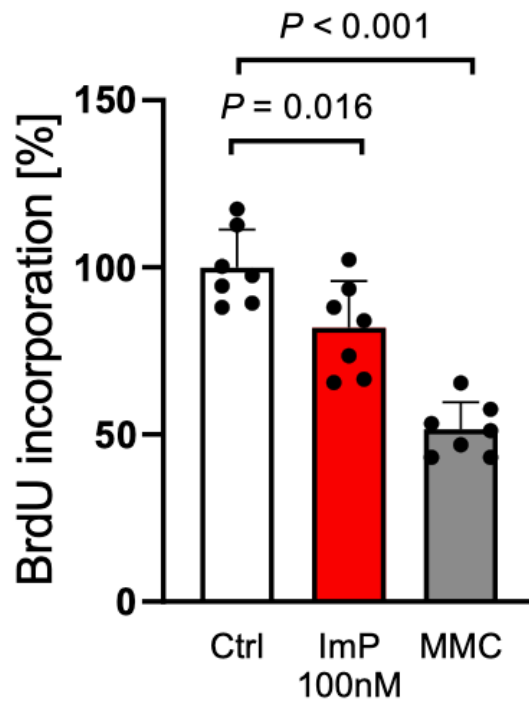


Figure S2: ImP significantly decreases endothelial cell proliferation. Cell proliferation rate of human aortic endothelial cells (HAECs) was assessed by BrdU incorporation assay upon 24 h of ImP treatment (100 nM). Mitomycin C (MMC; 10 μ g/ml) was used as positive control for proliferation inhibition. Data are shown as mean \pm SEM and were calculated by one-way ANOVA followed by the Bonferroni's post hoc analysis for multiple comparisons ($n = 7$).

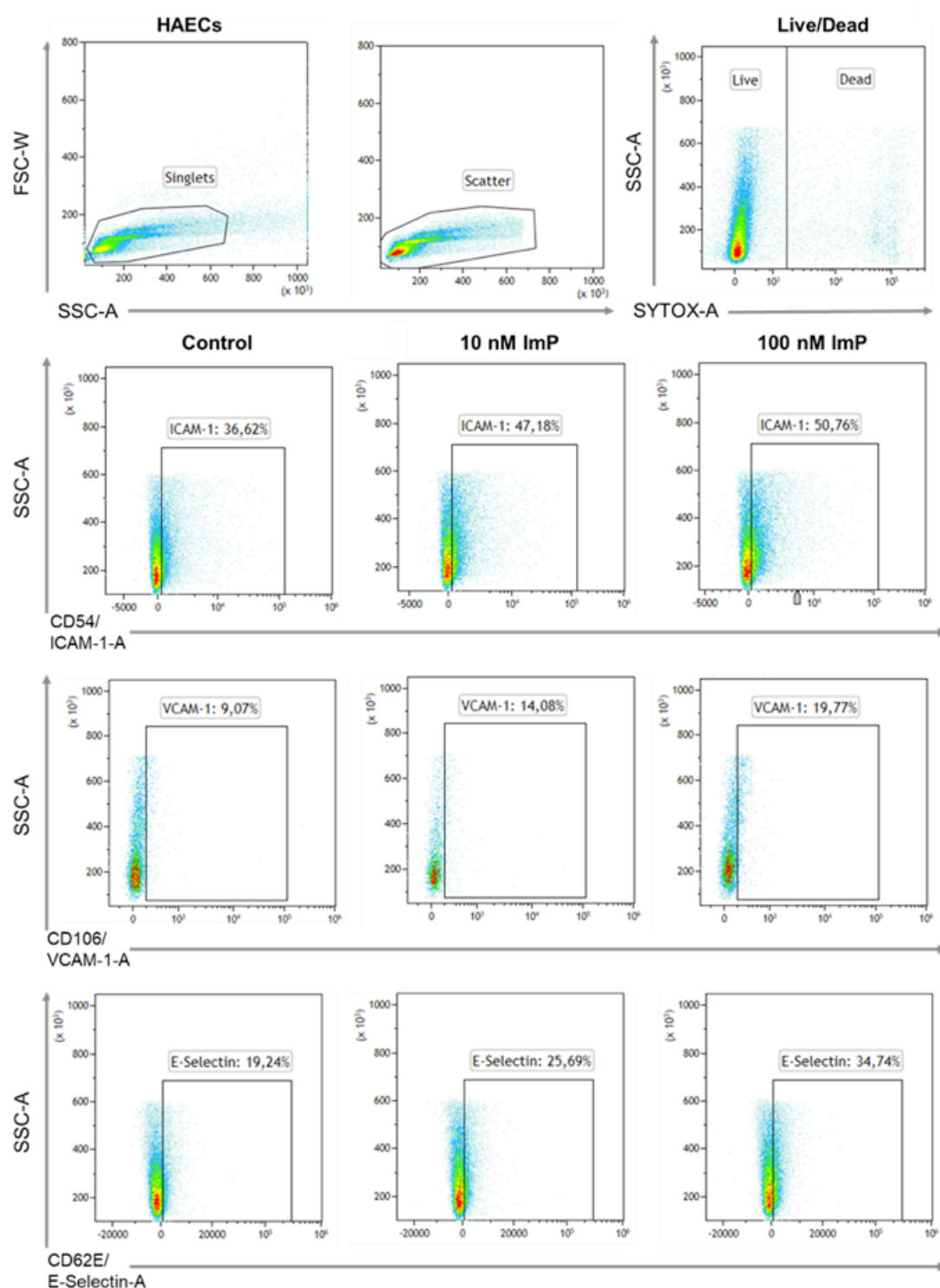


Figure S3: FACS gating strategy of pro-inflammatory adhesion molecules in endothelial cells. Representative dot plots demonstrate FACS gating strategy used to quantify pro-inflammatory cell adhesion molecules ICAM-1, VCAM-1 and E-selectin, in endothelial cells following treatment with or without ImP (24 h). Cell populations were initially gated for singlets based on forward scatter (FSC) and side scatter (SSC) properties. Debris was excluded, and a viability stain (Sytox) was applied to separate live cells from dead cells. The gated population of viable cells was then analyzed for ICAM-1, VCAM-1, and E-selectin expression, reported as a percentage of live endothelial cells.

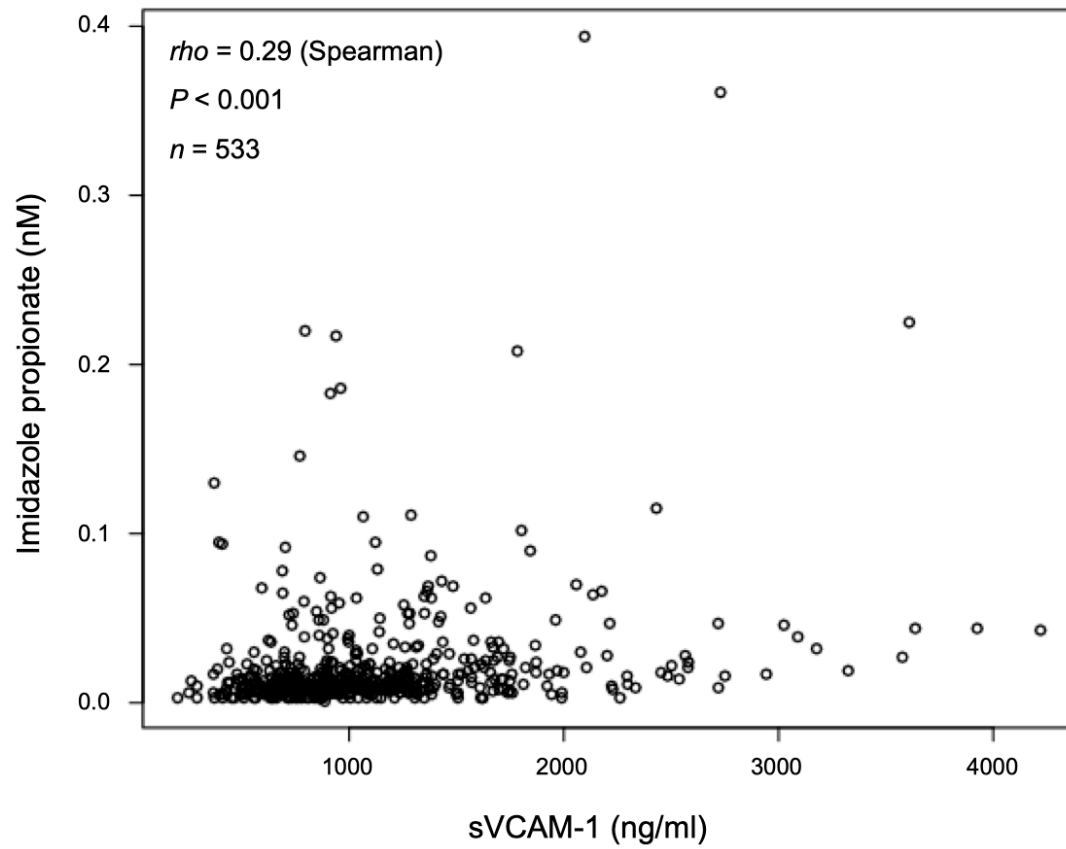


Figure S4: ImP significantly correlates with increased circulatory soluble VCAM-1 levels in blood of patients. Graph demonstrates the Spearman correlation between plasma levels of ImP (nM) and soluble VCAM-1 (ng/ml) from the GeneBank study supporting a putative link between ImP and VCAM-1 levels ($n = 533$).

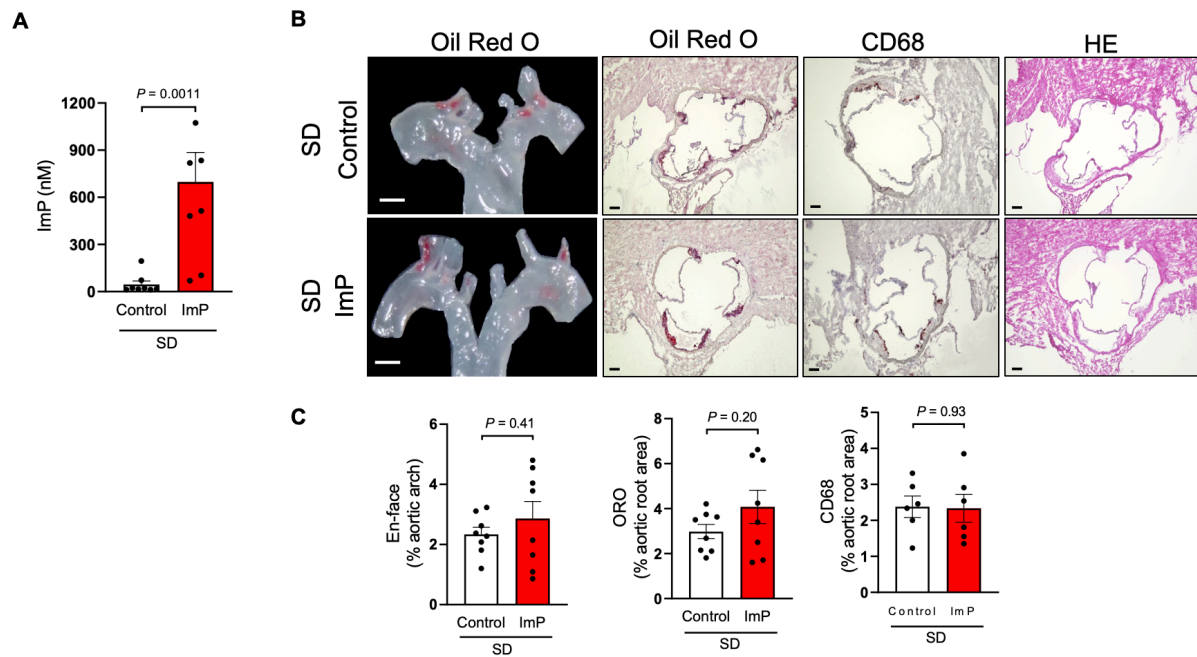


Figure S5: ImP did not significantly promote SCD-induced atherosclerosis in *Apoe*^{-/-} mice. **A**, Plasma levels of ImP in *Apoe*^{-/-} mice after 12 weeks treatment of a standard laboratory diet (SD) and exposure to control or ImP (800 µg/day). **B**, Representative images of Oil Red O (ORO)-stained *en face* aortic arch and of aortic root area stained with hematoxylin/eosin (HE), ORO and CD68 (scale bars represent 1mm for *en face* images or 100 µm for aortic root images). **B**, Quantification of plaque area in *en face* aortic arch and of total aortic root area as percentages. Data are shown as mean ± SEM and were calculated by two-tailed Mann-Whitney U-test (A) or unpaired two-tailed Student's *t*-test (C) ($n = 8$). Data with unequal variance (D: ORO staining of aortic roots) was assessed by the Welch *t*-test for two groups.

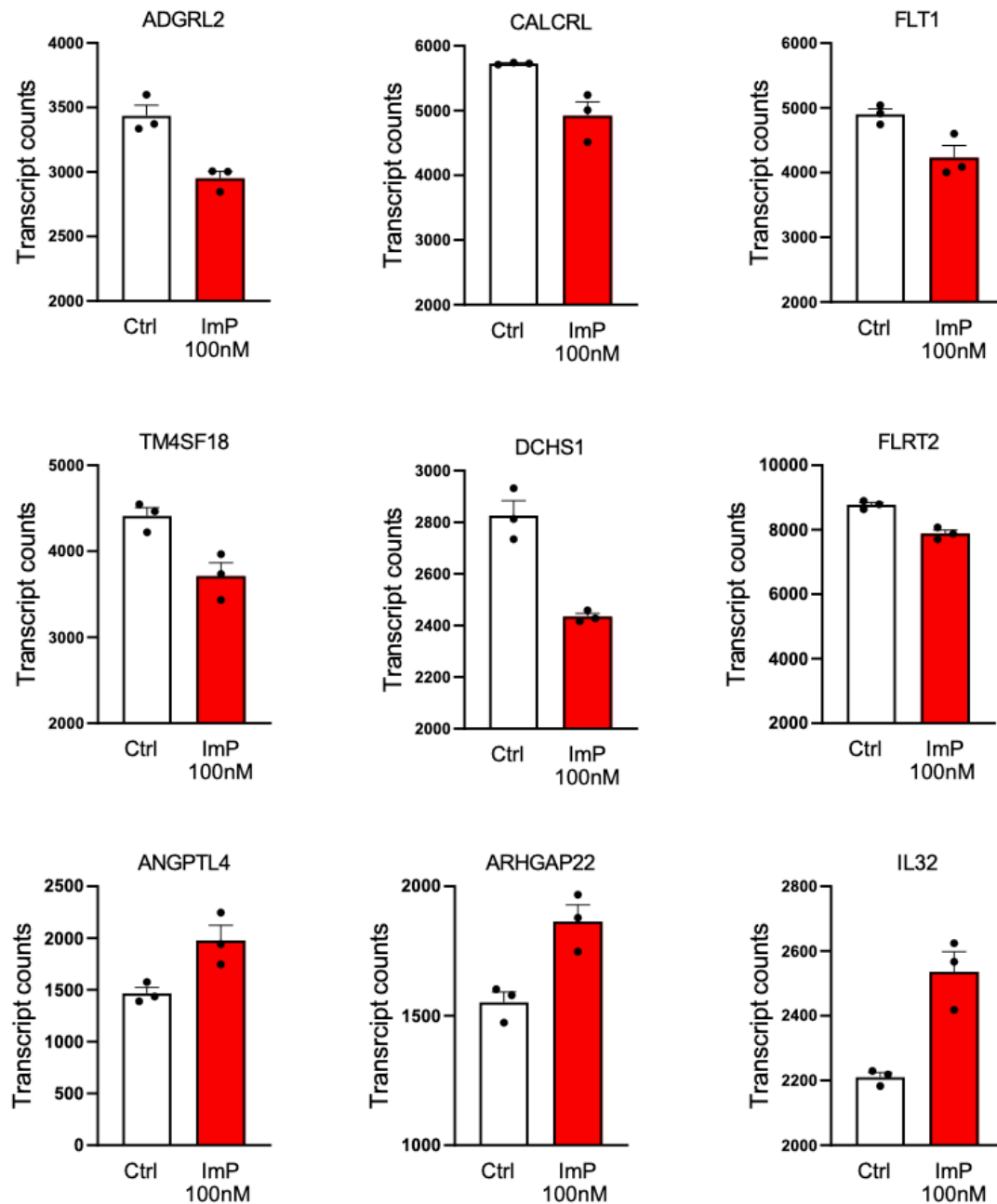


Figure S6: ImP downregulates a number of angiogenic genes in endothelial cells. Box plot charts illustrating key down- or upregulated genes from the RNA sequencing analysis involved in angiogenesis pathway including *ADGRL2*, *CALCRL*, *FLT1*, *TM4SF18*, *DCHS1*, *FLRT2*, *ANGPTL4*, *ARGHGAP22*, and *IL32* between the control and ImP group. Represented data are transcript counts differentially expressed in endothelial cells after treatment with and without ImP (100 nM) and from three independent experiments ($n = 3$).

Major Resources Table

In order to allow validation and replication of experiments, all essential research materials listed in the Methods should be included in the Major Resources Table below. Authors are encouraged to use public repositories for protocols, data, code, and other materials and provide persistent identifiers and/or links to repositories when available. Authors may add or delete rows as needed.

Animals (*in vivo* studies)

Species	Vendor or Source	Background Strain	Sex	Persistent ID / URL
<i>Mus Musculus</i>	Charles River Laboratories	C57BL/6J	Male	027C57BL/6
<i>Mus Musculus</i>	Charles River Laboratories	<i>Apoe</i> ^{-/-} (B6.129P2)	Male	01061900
<i>Mus Musculus</i>	Max Delbrück Center (MDC; Berlin, Germany)	<i>Cdh5-CreERT2</i> ^(+/-) / <i>FOXO1</i> ^{fl/fl}	Male	N/A
<i>Mus Musculus</i>	Max Delbrück Center (MDC; Berlin, Germany)	<i>Cdh5-CreERT2</i> ^(+/-) / <i>FOXO1</i> ^{fl/fl}	Male	N/A

Antibodies

Target antigen	Vendor or Source	Catalog #	Working concentration	Persistent ID / URL
AKT	Cell Signaling	9272	1:1000	RRID: AB_329827
CD106/ VCAM-1, APC	BioLegend	305810	1:100	RRID: AB_2214226
CD54/ICAM-1, Alexa Fluor700	BioLegend	353126	1:100	RRID: AB_2810561
CD62E/ E-Selectin, PE/Cyanine7	BioLegend	336016	1:100	RRID: AB_2800891
CD68	Abcam	ab53444	1 µg/ml	RRID: AB_869007
FOXO1	Cell Signaling	2880	1:2000 (WB) 1:100 (IF)	RRID: AB_2106495
GAPDH	Merck Millipore	MAB374	0.02 µg/ml	RRID: AB_2107445
Mouse IgG (H&L), HRP-linked	SouthernBiotech	1031-05	1:10000	RRID: AB_2795955
phospho-AKT (Ser473)	Cell Signaling	4060	1:1000	RRID: AB_2315049
phospho-FOXO1	Cell Signaling	9464	1:2000	RRID: AB_329842
PI3 Kinase Class II α	Cell Signaling	12402	1:2000	RRID: AB_2797900
Rabbit IgG (H&L), DyLight™ 594	Invitrogen	35560	2 µg/mL	RRID: AB_1185570
Rabbit IgG (H&L), HRP-linked	SouthernBiotech	4050-05	1:3000	RRID: AB_2794307
Rat IgG (H+L), Biotin-linked	DAKO	E0468	1:100	N/A
αSMA	Abcam	ab5694	1 µg/ml	RRID: AB_2223021

Oligonucleotides/ siRNA

siRNA Name	Sequence	Source / Repository	Catalog #
Control siRNA (scrambled)	not disclosed	Santa Cruz	sc-37007
FKHR/FOXO1	not disclosed	Santa Cruz	sc-35382

Cultured Cells

Name	Vendor or Source	Sex (F, M, or unknown)	Catalog #
Human aortic endothelial cells (HAECs)	Cell Applications	Male	304-05a
THP-1 monocytes	N/A	Unknown	N/A

Data & Code Availability

Description	Source / Repository	Persistent ID / URL
RNA sequencing data	European Nucleotide Archive (ENA)	PRJEB61803: https://www.ebi.ac.uk/ena/browser/view/PRJEB61803

Other

Description	Vendor or Source	Catalog #
ABC-HRP Kit for Peroxidase	Vector Laboratories	PK-4000
Acetylcholine (ACh)	Sigma-Aldrich	A6625
Acrylamide/Bis Solution (37.5:1)	Serva	10681.01
Ammonium persulfate	Thermo Scientific	BP179-100
Aprotinin	Serva	13718.01
Avidin-Biotin blocking solution	Vector Laboratories	SP-2001
Bovines Serumalbumin (BSA) Fraktion V	Carl Roth	8076.5
BrdU Cell Proliferation ELISA	Roche	11647229001
Bromophenol blue	Sigma-Aldrich	B5525
Calcein AM	R&D System	4892-010-01
Cobas, c.f.a.s.	Roche	3039773190
DAPI	R&D System	10236276001
Dil Cell-Labeling Solution	Invitrogen	V22885
DTT	Thermo Scientific	R0861
Dulbecco's Phosphate Buffered Saline	Sigma-Aldrich	D8537
EDTA	Carl Roth	8043.2
Endothelial Cell Growth Basal Medium-2	PromoCell	C-22111
Endothelial Cell Growth Medium-2	PromoCell	C-22111
Eosin Y solution	Sigma-Aldrich	HT110132
Evans blue dye	Sigma-Aldrich	E2129
Fetal bovine serum	Biowest	S181B-500
Geltrex™ BME Matrix	Gibco	A14133-02
Glycerol	Carl Roth	3783.3c
Glycine	Merck Millipore	104.201.100

Hemalum solution	Carl Roth	T865.2
High fat diet (HFD)	Ssniff GmbH	E15741
Human Fibronectin solutions	PromoCell	C-43060
Imidazole propionate (ImP)	Santa Cruz	sc294276; CAS 1074-59-5
Kaiser's glycerol gelatin	Merck Millipore	1.092.420.100
Leupeptin	Serva	51867.02
Lipofectamine RNAiMAX transfection kit	Invitrogen	13778075
NaCl	Carl Roth	HN00.3
NP-40	Sigma-Aldrich	74385
Oil Red O (ORO)	Alfa Aesar	A12989.22
Opti-MEM reduced serum Medium	Gibco	31985070
PageRuler Prestained Protein Ladder (Plus)	Thermo Scientific	26616; 26619
Penicillin-Streptomycin (10.000 U/ml)	Gibco	15140122
Phenylephrine (PE)	Sigma-Aldrich	P6126
Physiological salt solution (PSS) buffer	119 mM NaCl (Carl Roth; P029.3), 25 mM NaHCO ₃ (Merck; 6346.0500), 11.1 M D-Glucose (Carl Roth; 200-075-1), 1.2 mM KH ₂ PO ₄ (Carl Roth; 3904.1), 4.7 mM KCl (Carl Roth; 6781.1), 1.6 mM CaCl ₂ x H ₂ O (Merck; 1.02083.1000), 1.2 mM MgSO ₄ (Sigma-Aldrich; 20.809-4)	
Pierce™ BCA Protein Assay Kit	Thermo Scientific	23225
PMSF	Carl Roth	6367.1
Potassium physiological salt solution (KPSS) buffer	63.7 mM NaCl, 25 mM NaHCO ₃ , 11.1 mM D-Glucose, 1.2 mM KH ₂ PO ₄ , 100 mM KCl, 1.6 mM CaCl ₂ x H ₂ O, 1.2 mM MgSO ₄	
Recombinant human IGF-1	PeproTech	100-11
Recombinant human TNF-α	R&D System	210-TA
RPMI 1640 Medium	Gibco	A1049101
Skim milk powder	Serva	42590
Sodium dodecyl sulfate	Sigma-Aldrich	L5750
Sodium fluoride	Sigma-Aldrich	201154
Sodium nitroprusside (SNP)	Sigma-Aldrich	71780
Sodium orthovanadate	New England BioLabs	P0758S
Standard laboratory diet (SD)	Ssniff GmbH	E15000
SuperSignal™ West Dura Extended Duration Substrate	Thermo Scientific	34075
TEMED	AppliChem	APA1148.0025
Tissue-Tek OCT compound	Sakura	4583
Tris-base	Carl Roth	0188.4
Trypsin-EDTA (0,05 %), phenol red	Gibco	25300054
Tween 20	VWR	663684B
VCAM-1 Human ELISA Kit	Invitrogen	KHT0601

ARRIVE GUIDELINES

The ARRIVE guidelines (<https://arriveguidelines.org/>) are a checklist of recommendations to improve the reporting of research involving animals. Key elements of the study design should be included below to better enable readers to scrutinize the research adequately, evaluate its methodological rigor, and reproduce the methods or findings.

Study Design

Groups	Sex	Age (weeks)	Number (prior to experiment)	Number (after termination)	Littermates (Yes/No)
C57BL/6J (Control)	M	10	6	6	Combination
C57BL/6J (ImP)	M	10	6	6	Combination
<i>Apoe</i> ^{-/-} (Control + HFD)	M	19-20	8	8	Combination
<i>Apoe</i> ^{-/-} (ImP+ HFD)	M	19-20	8	8	Combination
<i>Apoe</i> ^{-/-} (Control + STD)	M	19-20	8	8	Combination
<i>Apoe</i> ^{-/-} (ImP + STD)	M	19-20	8	8	Combination
<i>Cdh5-CreERT2</i> ^(+/+) / <i>FOXO1</i> ^{fl/fl} (ImP control)	M	13	7	6	Combination
<i>Cdh5-CreERT2</i> ^(+/-) / <i>FOXO1</i> ^{fl/fl} (ImP)	M	13	7	7	Combination

Sample Size: Please explain how the sample size was decided. Please provide details of any a *prior* sample size calculation, if done.

The calculation of the sample sizes for a two-sided test are based on the estimated effect size with a significance level (α) of 0.05 and a power ($1-\beta$) of 0.8. The sample sizes were determined through a power analysis conducted with nQuery and nTerim 4.0, based on a similar study by Haghikia et al., 2022.⁵¹

Inclusion Criteria: All mice with appropriate genotypes were included in the experiments.

Exclusion Criteria: No mouse has been excluded from the study.

Randomization: Mice were randomly assigned to experimental groups, Control and Imidazole propionate (ImP). To assess atherosclerosis, *Apoe*^{-/-} mice were additionally randomly assigned to either a standard laboratory diet (SD) or high-fat-diet (HFD).

Blinding: Data analysis and statistics were performed by a blinded operator.