SUPPLEMENTAL MATERIAL

Indoleamine 2, 3-Dioxygenase 1 (IDO1) Deletion-Mediated Kynurenine Insufficiency in Vascular Smooth Muscle Cells Exacerbates Arterial Calcification

Running Title: *Tryptophan-Derived Kynurenine in Arterial Calcification*

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Supplemental Methods

Study population

This case-control study was conducted from March 2018 to January 2020 at Tungwah Hospital of Sun Yat-sen University, China. Of 198 participants with a diagnosis of chronic kidney disease (CKD), 10 participants who met the exclusion criteria, 10 participants who missed the blood collection, 22 patients without data from multidetector computed tomography (MDCT) scanning of the coronary artery, and 4 patients due to serum sample quality issue were excluded. The clinical study protocol followed ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Ethics Committee of the Tungwah Hospital of Sun Yat-sen University (2017DHLL017). All participants gave written informed consent before entering the study.

The diagnosis of CKD was based on an estimated glomerular filtration rate (eGFR) decline of longer than 6 months. CKD staging followed guidelines based on Chinese population.[44,45] CKD stage 1 to 2 was defined as an albumin/creatinine ratio > 14 mg/g in men and > 20 mg/g in women, with an eGFR ≥ 60 mL/min per 1.73 m^2. CKD stage 3 to 5 was defined as an eGFR < 60 mL/min per 1.73 m^2 before dialysis. The exclusion criteria were: (1) age <18 or ≥ 90 years; (2) pregnancy; (3) administration of any phosphate binder, vitamin D analog, or corticosteroid medications within 3 months before recruitment; (4) acute renal failure; (5) uncontrolled hyperlipidemia, and (6) history of hepatic disease or neoplastic disease.

The CT images were reconstructed using slices of 1 mm thickness to evaluate coronary artery calcification. Agatston scores of the MDCT images were quantified following standard criteria by two independent technicians blinded to the patients' information, and using Siemens Syngo CT Workplace software. In asymptomatic, symptomatic patients or patients with metabolic diseases, a coronary artery calcification (CAC) score of zero predicts a low risk of cardiovascular events or

all-cause mortality in the medium and long term.[46-48] In the cohort, the median of CAC Agatston score was 83.18 (39.76, 372.5), and 50 (32.89%) of patients were diagnosed with diabetes complication. Therefore, presence of calcification burden was defined as an Agatston score > 0. All subjects are ethnical Chinese (Han), and the multiple linear regression analyses of clinical factors and CAC Agatston scores were adjusted for age and sex.

Animal experiments

The animal experiments were performed following protocols approved by the Georgia State University Institute Animal Care and Use Committee. Specific-pathogen-free mice were raised at the animal facility of Georgia State University in controlled, identical temperature, humidity, and 12-hour light-dark cycle conditions. They had free access to sterilized water and the assigned diet. *Ido1*$^{-/-}$ (stock no. 005867), *Myh11*^{Cre} (stock no. 007742), *lysM*^{Cre} (stock no. 004781), and *Apoe*$^{-/-}$ (stock no. 002052) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). *Ido1*$^{fl/fl}$ mice were obtained from the Wellcome Trust Sanger Institute (Hinxton, UK). *Ido1*$^{-/-}$ mice were bred to *Apoe*$^{-/-}$ mice. *Ido1*$^{fl/fl}$ *Myh11*^{Cre} mice were achieved by crossbreeding *Ido1*$^{fl/fl}$ mice with *Myh11*^{Cre} mice. Specific lineage mice were obtained by crossbreeding *Ido1*$^{fl/fl}$ mice with previously established *Apoe*$^{-/-}$ *Myh11*^{Cre} and *Apoe*$^{-/-}$ *lysM*^{Cre} mice.[21,49]

For the diet-induced atherosclerotic calcification model, eight-week-old male or female mice were randomly provided with regular chow diet for 24 weeks as the control group, or diet that had a nutritional profile similar to the Western diet (0.21% cholesterol, 40 kcal% fat, and 43 kcal% carbohydrate; D12079B, Research Diets) for 24 weeks as the experimental group.[21]

For kynurenine supplementation in vivo, after a 16-week Western diet challenge, *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$ *Ido1*$^{-/-}$ mice were injected with kynurenine (100 mg/kg) or vehicle via the intraperitoneal route every 48 hours for 8 weeks, along with continued consumption of Western diet. Kynurenine was

first dissolved in dimethyl sulfoxide (DMSO) and then diluted further in 10% Captisol solution (Captisol Technology).[10] Thirty-six hours after the last kynurenine injection, the mice were fasted for another 12 hours before euthanasia to assure gastrointestinal emptying, in order to reduce the effect of food intake on serum tryptophan-kynurenine levels.

Blood was collected, whole aortas, including the brachiocephalic and femoral arteries, were removed for further analyses. Fasting blood glucose levels were detected using an autoanalyzer. Serum levels of total cholesterol and triglyceride were measured using specific reagents (Infinity™, Thermo Fisher Scientific). Serum levels of calcium were detected using a commercial kit (ab102505, Abcam) according to the manufacturer's instructions.

Chemicals and antibodies

L-ascorbic acid (A4544), β-glycerophosphate (G9422), perchloric acid (244252), ammonium hydroxide (09859), MG132 (M7449), cycloheximide (01810), chloroquine (C6628), DAPI (D9564), 3-hydroxykynurenine (H1771), and anthranilic acid (A89855) were obtained from Sigma-Aldrich (MO, USA). Kynurenic acid (sc-202683), 3-hydroxyanthranilic acid (sc-216460), xanthurenic acid (sc-258335), quinolinic acid (sc-203226), and puromycin (sc-108071) were purchased from Santa Cruz Biotechnology (TX, USA). Kynurenine (11305) was ordered from Cayman (MI, USA). The oxidized low-density lipoprotein (oxLDL, L34357) was ordered from Thermo Fisher Scientific (MA, USA).

Antibodies against RUNX2 (12556S), ubiquitin (3936S), calponin1 (17819S), SRY-box transcription factor 9 (SOX9, 82630S), mouse IgG (5873S), and rabbit IgG (2729S) were purchased from Cell Signaling Technology (MA, USA). Antibody against RUNX2 (D130-3) for immunoprecipitation was purchased from MBL (MA, USA). Antibodies against alpha smooth

muscle actin (α-SMA, ab7817), smooth muscle 22 alpha (SM22α, ab14106), and osteopontin (OPN, ab8448) were purchased from Abcam (Cambridge, UK). Antibody against IDO1 (MABF850) was purchased from Millipore (MA, USA). The collagen I alpha1 (COL1A1, sc-293182), osteocalcin (OCN, sc-390877), aryl hydrocarbon receptor (AhR, sc-133088), cullin 4B (CUL4B, sc-377188), msh homeobox 2 (MSX2, sc-365232), GAPDH (sc-32233), and β-Actin (sc-47778) antibody were purchased from Santa Cruz Biotechnology.

LC-MS/MS and HPLC conditions

Before dialysis, a baseline median cubital venous blood sample was collected from each CKD patient after an overnight fast. The serum was then isolated for subsequent analyses. Methanol and 5-hydroxyindole-2-carboxylic acid solution were used for protein precipitation. Analyses of the clinical samples were performed using a Shimadzu LC–MS/MS system (8060) equipped with LabSolutions software. A Hypersil™ BDS C18 50×2.1 mm (28105-052130, Thermo Fisher Scientific) column was used for the chromatographic separation. Solvent A (deionized water with 0.1% formic acid) and solvent B (methanol) were used as the mobile phases with flow rate of 0.3 mL/min under a gradient elution. The measurement was performed using a positive-ion multiple reaction monitoring method for the analytes. The optimal parameters were: capillary voltage, 3.0 kV; nebulizing gas flow rate, 3.0 L/min; heating gas flow rate, 10.0 L/min; drying gas flow rate, 10.0 L/min; source temperature, 300°C; heat block temperature, 400°C.

The aorta tissue homogenates and serum from the mice, and supernatant media and lysates from cultured cells were prepared for determination of tryptophan and kynurenine concentrations using HPLC, as previously described.[10] In brief, samples were deproteinized by mixing with equal volumes of precooled diluted perchloric acid (12%). Detection was performed using an autosampler (Waters, MA) and 250×4.6 mm C18 column (00G-4447-E0, Phenomenex). The

mobile phase of 20 mmol sodium acetate at pH 4.5 were filtered (0.45 μm) before use. The effluent was monitored at 280 nm (tryptophan) and 360 nm (kynurenine) using a UV detector with 1 mL/min flow rate. Tryptophan peak is identified around 8.3 min retention time at 280 nm, and kynurenine peak is identified around 4.8 min at 360 nm, both representing typical chromatographic separation. IDO1 activity was reflected as Kyn/Trp ratio.

Immunofluorescence

Cryosections (8 μm) were processed as previously reported.[49] Slides were washed with deionized water to remove the optimum cutting temperature compound. Slides were then fixed in ice-cold acetone for 5 minutes, and then incubated with 5% serum for 30 minutes in a humidified chamber to block non-specific binding. Slides were incubated with antibodies against RUNX2, COL1A1, or α-SMA at 4°C overnight and then exposed to fluorescent label conjugated secondary antibodies (Alexa Fluor®, Invitrogen). Nuclei were counter-stained using DAPI. Images were captured using a microscope (IX73, OLYMPUS) and were quantified using with ImageJ (National Institutes of Health, USA). Results were expressed as the ratio of relative florescence intensity compared with the controls. All quantifications were performed by two observers without knowledge of the identities of samples.

Cell and aortic ring isolation

Primary VSMCs isolated from aortas of eight-week-old male mice were cultured in supplemented 1640 medium (Lonza, USA), as previously described.[49] Human coronary VSMCs were purchased from the American Type Culture Collection (MD, USA) and maintained in M231 medium (Life Technology, USA) containing 5% fetal bovine serum (FBS) (GIBCO, USA). VSMCs between passage numbers 3 to 6 were used for the experiments. Thoracic aortas were dissected under sterile

conditions to remove fat tissue, adventitia, and endothelium. They were then cut into 5-mm rings and cultured in Dulbecco's modified eagle's medium (GIBCO, USA) containing 10% FBS.

Cellular transfections

Cells were transfected with control siRNA (sc-37007), IDO1 siRNA (sc-45939), AhR siRNA (sc-29654), or CUL4B siRNA (sc-37572) purchased from Santa Cruz Biotechnology, in combination with Lipofectamine RNAiMAX Reagent (13778150, Invitrogen), following the manufacturer's protocol.

Plasmids encoding RUNX2 (RC213097, Origene), AhR (HG10456-CY, SinoBiological), or CUL4B (19922, Addgene) were transfected into VSMCs by electroporation using the P1 Primary Cell 4D-Nucleofector X Kit L from Lonza (MD, USA) following the manufacturer's instructions. VSMCs or aortic rings were infected with recombinant lentiviral particles carrying control short hairpin RNA (shRNA) (sc-108080), RUNX2 shRNA (sc-37145-V; sc-37146-V), or IDO1 shRNA (sc-45939-V) purchased from Santa Cruz Biotechnology, according to the manufacturer's instructions. Stable colonies were selected using 7-day 5 μg/mL puromycin challenges.

In vivo calcification evaluation

Areas of calcification were identified using von Kossa stain (KTVKO, American MasterTech Scientific) and analyzed using ImageJ in a blinded manner. Briefly, sections were immersed with 5% silver nitrate buffer and exposed to ultraviolet light for 30 minutes, rinsed in deionized water, incubated with 5% sodium thiosulfate, and were then exposed to nuclear fast red stain. Calcium crystals stained brown, cytoplasm stained pink, and nuclei stained red. Calcified spots within the medial aortic layer were evaluated based on percentage of calcification lesion size (% of plaque area).

Released calcium in the homogenate of the medial aortic layer was quantified using the Calcium Assay Kit (ab102505, Abcam) in compliance with the manufacturer's protocol. Calcium content was calibrated by the weight of aorta. Alkaline phosphatase (ALP) activity in aortas were determined using a commercial kit (DALP-250, QuantiChrom).

In vitro calcification evaluation

VSMCs or aortic rings were exposed to a combination of 0.25 mmol/L L-ascorbic acid and 10 mmol/L β-glycerophosphate to boost calcification formation.[21] Mineralization of cells was determined using alizarin red stain (AA42746AP, Thermo Fisher Scientific). A red color indicated a positive stain result. Calcium content and ALP activity of aortic rings were analyzed as described above.

Quantitative real time polymerase chain reaction (RT-PCR)

TRIzol (15596-026, Invitrogen) was used for total RNA extraction from VSMCs or aorta tissues, and an iScript cDNA Synthesis Kit (1708890, Bio-Rad) was used for reverse transcription. PCR was performed in triplicate using iQ SYBR Green supermix and a CFX96 Touch real-time system (Bio-Rad, USA). The primers used were: *Gapdh* F-5'-AACTTTGGCATTGTGGAAGG-3', R-5'-ACACATTGGGGGTAGGAACA-3'; *Ido1* F-5'-CCCACACTGAGCACGGACGG-3', R-5'-TTGCGGGGCAGCACCTTTCG-3'; *Runx2* F-5'-CGGCCCTCCCTGAACTCT-3', R-5'-TGCCTGCCTGGGATCTGTA-3'; GAPDH F-5'-GGAGTCAACGGATTTGGT-3', R-5'-GTGATGGGATTTCCATTGAT-3'; RUNX2 F-5'-CTTCACAAATCCTCCCCAG-3', R-5'-GAATGCGCCCTAAATCA-3'. The cycle threshold was normalized based on housekeeping gene results.

Immunoprecipitation and western blot analysis

Before immunoprecipitation, VSMCs were pretreated with MG132 for 6 hours to prevent proteasomal degradation. Samples were lysed in non-denaturing buffer (20 mmol/L Tris-HCl pH 7.5, 150 mmol/L NaCl, 10% glycerol, 1% Triton X-100) containing protease inhibitor cocktail (5871S, Cell Signaling Technology). Immune complexes were precipitated with the appropriate primary antibody overnight and then incubated with a slurry of IgG magnetic beads (S1431S, NEB) for another 2 hours at 4°C with gentle rotation. Beads were suspended in 2×Laemmli buffer (BP-111NR, Boston Bioproducts) and boiled briefly for complete denaturation.

Cytoplasmic and nuclear fractions were prepared as previously described.[50] Cellular lysates or aortic tissue homogenates were examined using western blot analysis, as previously described.[49] Individual bands were quantified based on integrated density, using ImageJ software.

Statistical analyses

All results were expressed as mean ± standard error of the mean (SEM), median (25th-75th quartiles) for continuous variables, or n (%) for categorical variables, respectively. The Shapiro-Wilk test or D'Agostino-Pearson test was performed to assess data normality. The equality of group variances was assessed using *F* test or Brown-Forsythe test. For comparisons between two groups, significance was assessed using unpaired two-tailed Student's *t*-tests, unpaired *t*-test with Welch's correction, or nonparametric Mann-Whitney *U* test. For comparisons among multiple groups, analysis of variance (ANOVA) or Welch's ANOVA test accompanied by Tukey's post hoc test (equal variances assumed), Dunnett's post hoc test, or Tamhane's T2 post hoc test (equal variances not assumed) was performed. Correlation was assessed using Spearman's rank correlation coefficient. $P < 0.05$ was considered statistically significant. Statistical analyses were performed using SPSS version 28.0 (IL, USA) or GraphPad Prism 8.0 (CA, USA).

Supplemental Tables

Supplemental Table 1. Baseline characteristics of CKD patients with or without CAC.

Characteristics	Non-CAC (n = 71)	CAC (n = 81)	*P* value
Demographic characteristics			
Male (%)	49 (69.0)	48 (59.2)	0.2389
Age (year)	58.4 ± 1.8	67.2 ± 1.4	**0.0001**
BMI (kg/m^2)	23.9 ± 0.6	23.2 ± 0.4	0.3073
SBP (mmHg)	139.8 ± 2.5	142.7 ± 2.6	0.4230
DBP (mmHg)	82.1 ± 1.4	79.4 ± 1.7	0.2157
Plasma biochemical characteristics			
K (mmol/L)	4.31 ± 0.08	4.26 ± 0.07	0.6535
Ca (mmol/L)	2.18 (2.04 - 2.29)	2.17 (2.07 - 2.27)	0.8546
Pi (mmol/L)	1.35 (1.04 - 1.74)	1.25 (1.02 - 1.66)	0.5863
GLU (mmol/L)	5.39 (4.34 - 6.35)	5.18 (4.50 - 7.33)	0.8402
BUN (mmol/L)	12.59 (5.09 - 21.15)	16.35 (9.61 - 23.13)	0.0625
CREA (μmol/L)	457.8 (135.8 - 903.5)	576.1 (192.1 - 1030)	0.1169
eGFR (mL/min·per 1.73m^2)	12.44 (5.19 - 48.42)	8.20 (4.23 - 25.50)	0.0894
URCA (μmol/L)	395.2 ± 15.45	390.8 ± 14.56	0.254
ALB (g/L)	36.58 ± 0.71	35.02 ± 0.59	0.0919
ALT (U/L)	11 (7 – 17.6)	11 (7 - 17)	0.7472
AST (U/L)	16 (12 - 22)	15 (11 - 19)	0.1220
ALP (U/L)	71 (55 - 90)	80 (62 - 122)	**0.0277**
CHOL (mmol/L)	4.01 (3.31 - 4.68)	3.95 (3.22 - 4.84)	0.9684
TG (mmol/L)	1.37 (0.90 - 2.17)	1.37 (0.94 - 2.13)	0.8270
HDL-C (mmol/L)	1.03 (0.77 - 1.42)	0.99 (0.75 - 1.37)	0.7578
LDL-C (mmol/L)	2.31 (1.96 - 3.07)	2.49 (1.51 - 3.31)	0.9435

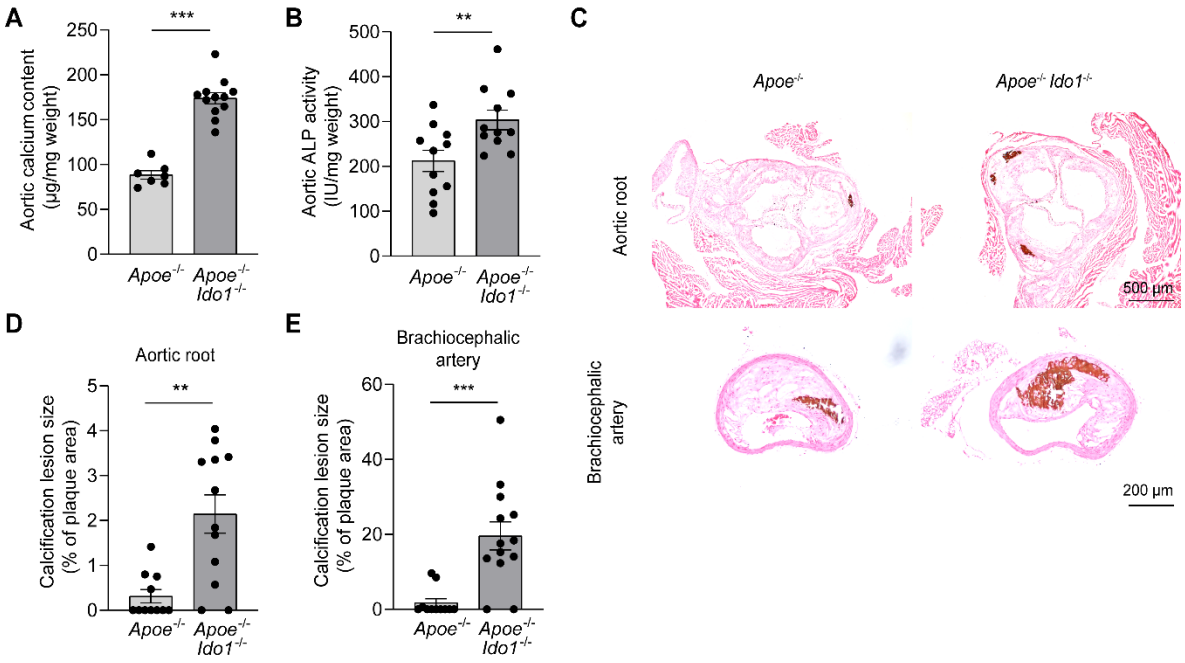
Values are expressed as mean ± SEM or median (25% - 75% percentile) for continuous variables or n (%) for categorical variables, respectively. Statistical significance was assessed using Student's *t*-test or nonparametric Mann-Whitney test. CAC, coronary artery calcification; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; K, potassium; Ca, calcium; Pi, phosphate; GLU, glucose; BUN, blood urea nitrogen; CREA, creatinine; eGFR, estimated glomerular filtration rate; URCA, uric acid; ALB, albumin; ALT, alanine transaminase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; CHOL, total cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol.

Supplemental Table 2. Multiple linear regression analysis of association of clinical factors and CAC Agatston Score adjusted for age and sex.

Variable	β	95% CI	*P*
BMI (kg/m^2)	0.079	-0.266 to 0.424	0.650
SBP (mmHg)	0.196	-1.573 to 1.964	0.827
DBP (mmHg)	-0.588	-2.783 to 1.608	0.597
Pi (mmol/L)	-0.203	-0.665 to 0.259	0.386
GLU (mmol/L)	2.084	-0.476 to 4.644	0.109
eGFR ($mL/min/1.73m^2$)	-0.243	-0.811 to 0.326	0.399
URCA (μmol/L)	-0.015	-0.068 to 0.038	0.573
ALB (g/L)	-0.025	-0.275 to 0.226	0.847
ALT (U/L)	0.073	-0.072 to 0.218	0.318
ALP (U/L)	0.026	0.007 to 0.046	**0.009**
CHOL (mmol/L)	0.241	-1.044 to 1.526	0.711
TG (mmol/L)	-0.500	-1.546 to 0.547	0.346
HDLC (mmol/L)	-1.012	-4.506 to 2.481	0.567
Kyn (μg/mL)	-14.272	-17.275 to -11.269	**< 0.001**

BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; Pi, phosphate; GLU, glucose; eGFR, estimated glomerular filtration rate; URCA, uric acid; ALB, albumin; ALT, alanine transaminase; ALP, alkaline phosphatase; CHOL, total cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein cholesterol; Kyn, kynurenine. In consideration of the variation among clinical variables, the value of CAC scores, SBP, DBP, and URCA were took a square root transformation for analysis, and the regression residuals are approximately normally distributed.

Supplemental Figures and Figure Legends



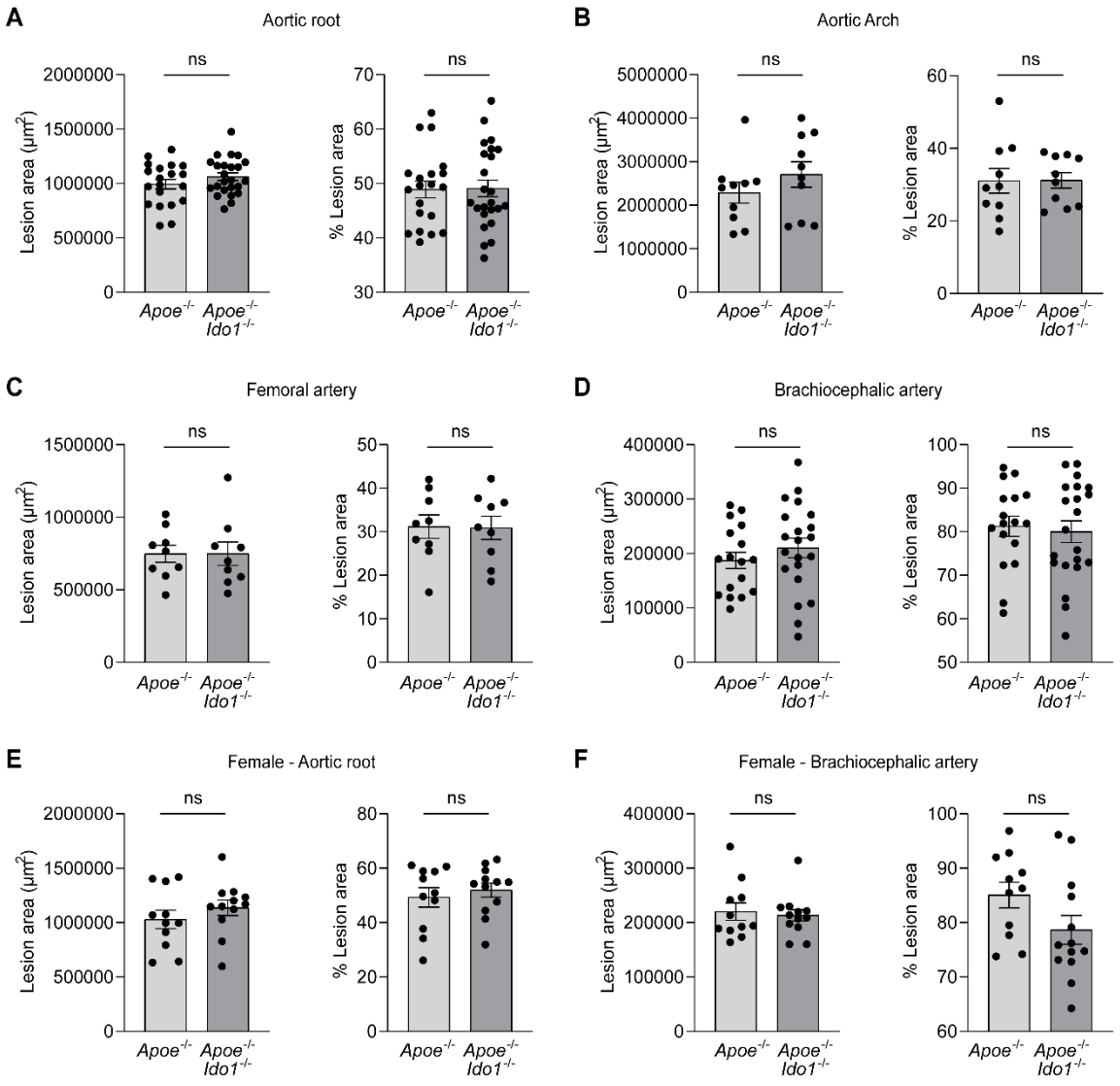
Supplemental Figure 1. IDO1 depletion promotes atherosclerotic calcification without gender bias

Female *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$ *Ido1*$^{-/-}$ mice were fed with Western diet for 24 weeks.

A and **B**, Biochemical measurement of aortic calcium content (**A**) and serum ALP activity (**B**) levels in female *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$ *Ido1*$^{-/-}$ mice (n = 7-12).

C-E, Representative von Kossa staining (**C**) and quantification performed in serial sections of aortic root (**D**) and brachiocephalic artery tissues (**E**) of female *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$ *Ido1*$^{-/-}$ mice (n = 11-13). Scale bars: 500 μm for aortic root, 200 μm for brachiocephalic artery.

Results are presented as mean ± SEM. *P* values are assessed using two-tailed unpaired Student's *t*-test for **A** and **B**, Mann Whitney test for **D** and **E**. $^{**}P < 0.01$, $^{***}P < 0.001$.



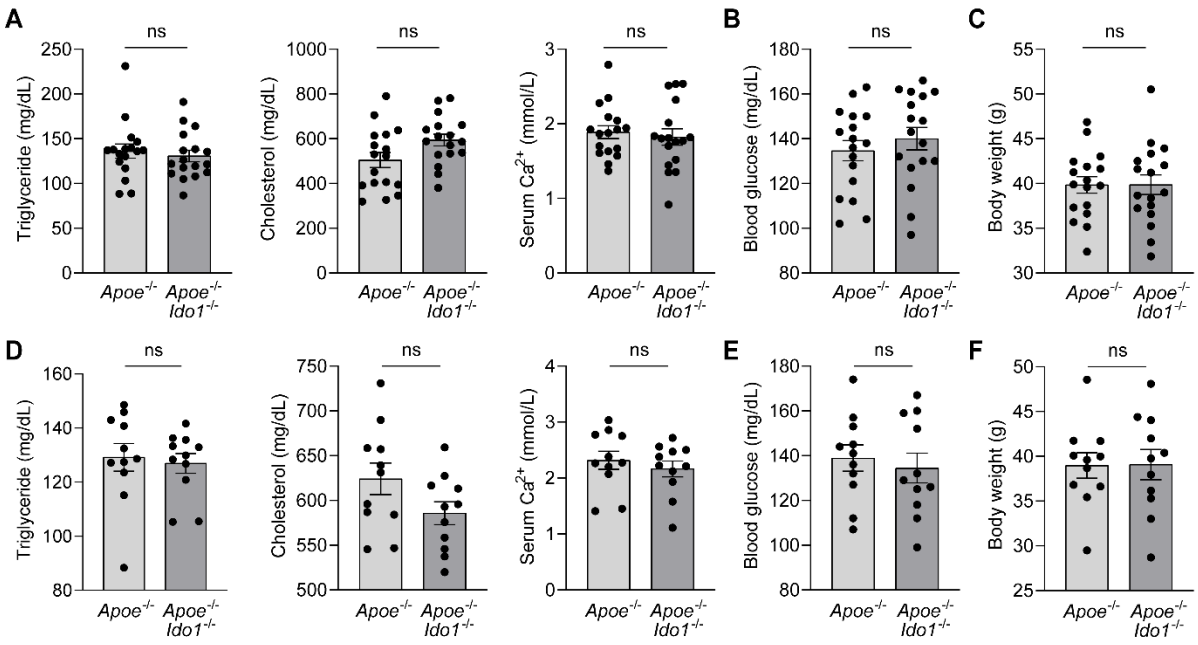
Supplemental Figure 2. IDO1 deficiency has no effect on plaque formation in situ

Male and female *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$ *Ido1*$^{-/-}$ mice were fed with Western diet for 24 weeks.

A-D, Quantification of atherosclerotic lesion size and percentage of lesion size within lumen area in aortic root (**A**), aortic arch (**B**), femoral artery (**C**), and brachiocephalic artery (**D**) tissue sections of male *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$ *Ido1*$^{-/-}$ mice (n = 20-24 for **A**, n = 9-10 for **B** and **C**, n = 17-21 for **D**).

E and **F**, Quantification of atherosclerotic lesion size and percentage of lesion size within lumen area in aortic root (**E**) and brachiocephalic artery (**F**) tissue sections of female *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$ *Ido1*$^{-/-}$ mice (n = 11-13).

Results are presented as mean ± SEM. *P* values are assessed using two-tailed unpaired Student's *t*-test. ns indicates $P > 0.05$.



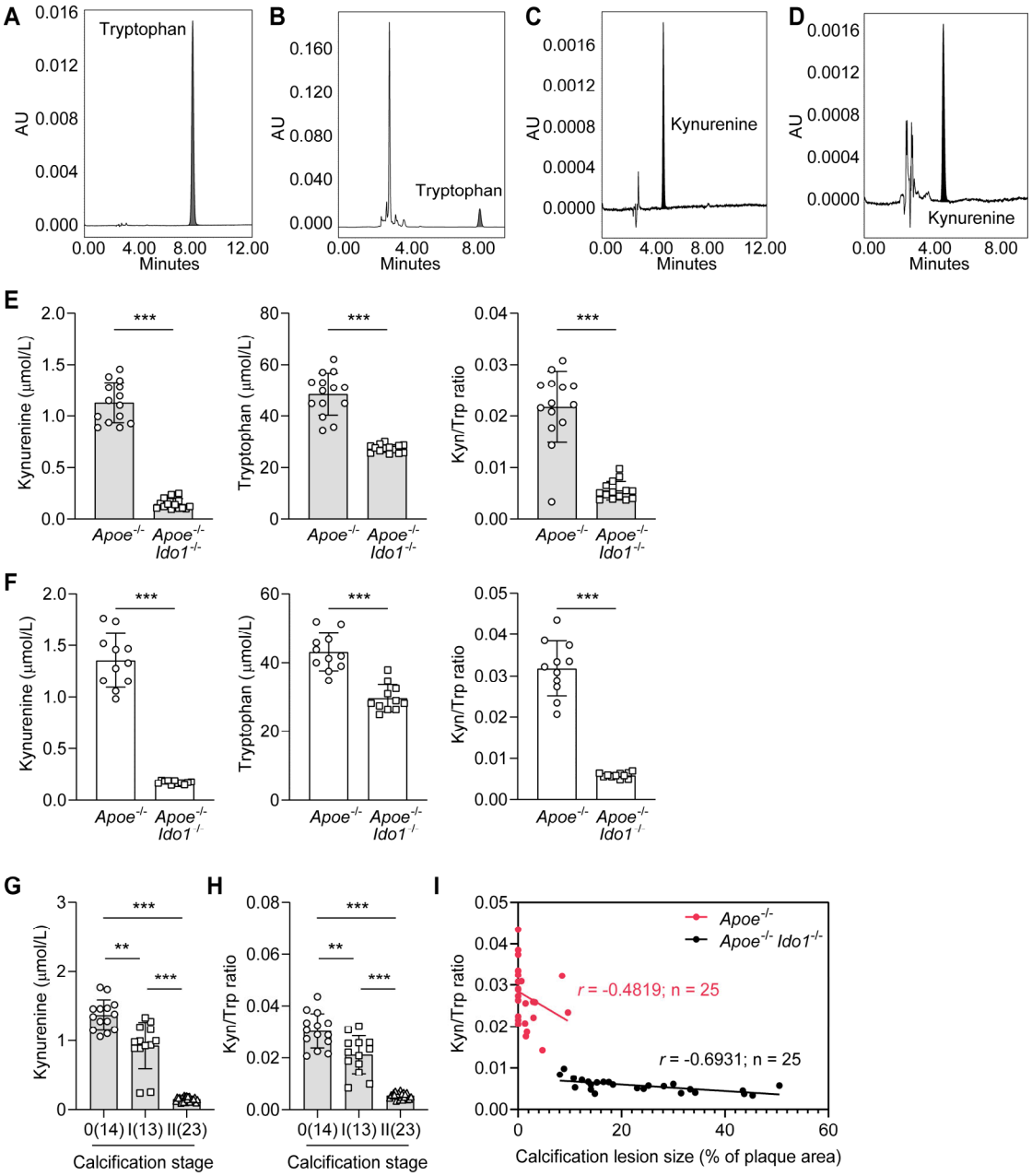
Supplemental Figure 3. IDO1 deficiency has no effect on metabolic parameters

Male and female *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$ *Ido1*$^{-/-}$ mice were fed with Western diet for 24 weeks.

A-C, Biochemical measurement of serum triglyceride (**A**), cholesterol (**A**), calcium (**A**), blood glucose (**B**), and body weight (**C**) of male *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$ *Ido1*$^{-/-}$ mice (n = 17).

D-F, Serum triglyceride (**D**), cholesterol (**D**), calcium (**D**), blood glucose (**E**), and body weight (**F**) detected in female *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$ *Ido1*$^{-/-}$ mice (n = 11).

Results are presented as mean ± SEM. *P* values are assessed using two-tailed unpaired Student's *t*-test. ns indicates $P > 0.05$.



Supplemental Figure 4. Serum kynurenine level is negatively associated with atherosclerotic calcification

Male and female *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$ *Ido1*$^{-/-}$ mice were fed with Western diet for 24 weeks.

A-**D**, Representative HPLC chromatograms of standard solution of tryptophan (**A**) and *Apoe*$^{-/-}$ serum sample (**B**) at 280 nm, standard solution of kynurenine (**C**) and *Apoe*$^{-/-}$ serum sample (**D**) at 360 nm.

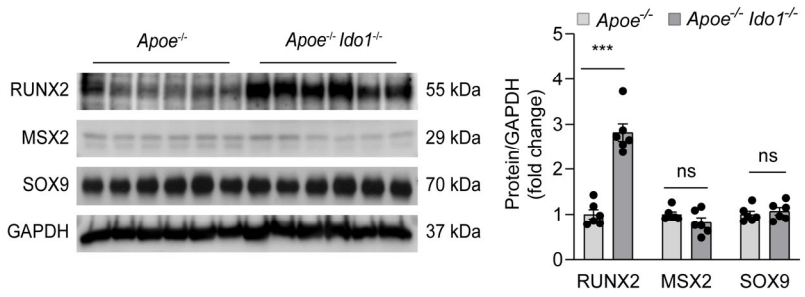
E and **F**, Serum kynurenine, tryptophan levels, and Kyn/Trp ratios detected in male (**E**) or female (**F**) *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$ *Ido1*$^{-/-}$ mice (n = 11-14).

G, Serum levels of kynurenine in the indicated groups of mice. The mice from **E** and **F** were classified as three groups based on percentage of calcification lesion size within atherosclerotic lesions in brachiocephalic artery tissues. 0: 0% (n = 14), I: 0–10% (n = 13), II :> 10% (n = 23).

H, Serum Kyn/Trp ratios in the indicated groups of mice same as **G**.

I, Spearman's rank correlation coefficient analysis for serum Kyn/Trp ratios correlated with the percentages of calcification lesion sizes within atherosclerotic lesion in mice same as **G**.

Results are presented as mean ± SEM. *P* values are assessed using two-tailed unpaired *t*-test with Welch's correction for **E** and **F**. Welch's ANOVA test with Tamhane's T2 post hoc test is used for **G** and **H**. Spearman's rank correlation coefficient is used for **I**. $^{**}P < 0.01$, $^{***}P < 0.001$.

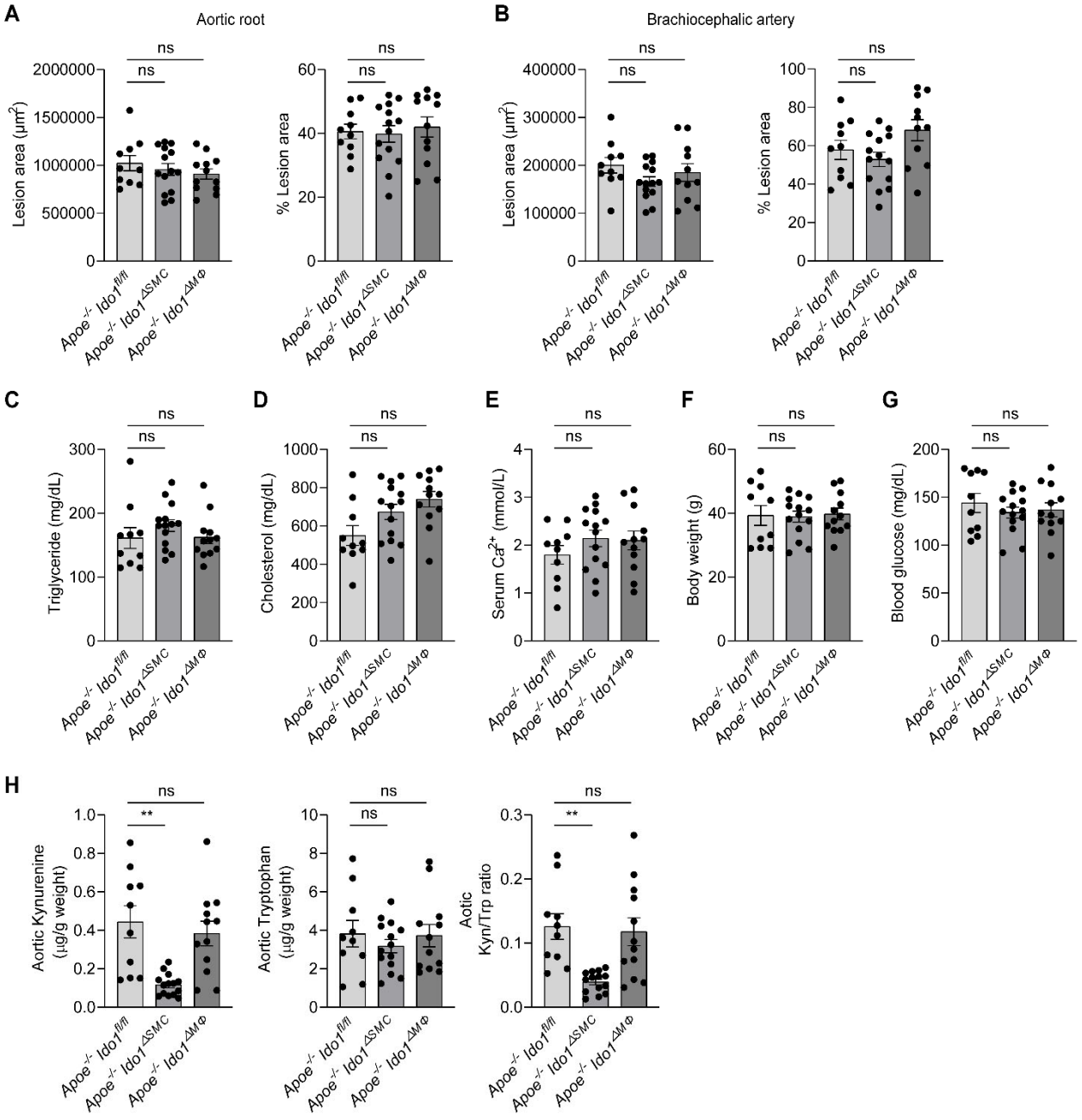


Supplemental Figure 5. Calcification-related factors expression in mice with IDO1 depletion

Apoe$^{-/-}$ and *Apoe*$^{-/-}$ *Ido1*$^{-/-}$ mice were fed with Western diet for 24 weeks.

Western blot analysis of RUNX2, MSX2, and SOX9 expression levels in whole aorta lysates from *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$ *Ido1*$^{-/-}$ mice (n = 6).

Results are presented as mean ± SEM, and analyzed using two-tailed unpaired Student's *t*-test. ***$P < 0.001$, ns indicates $P > 0.05$.



Supplemental Figure 6. IDO1 specific deletion in VSMCs or macrophages has no effect on plaque formation in situ and metabolic parameters

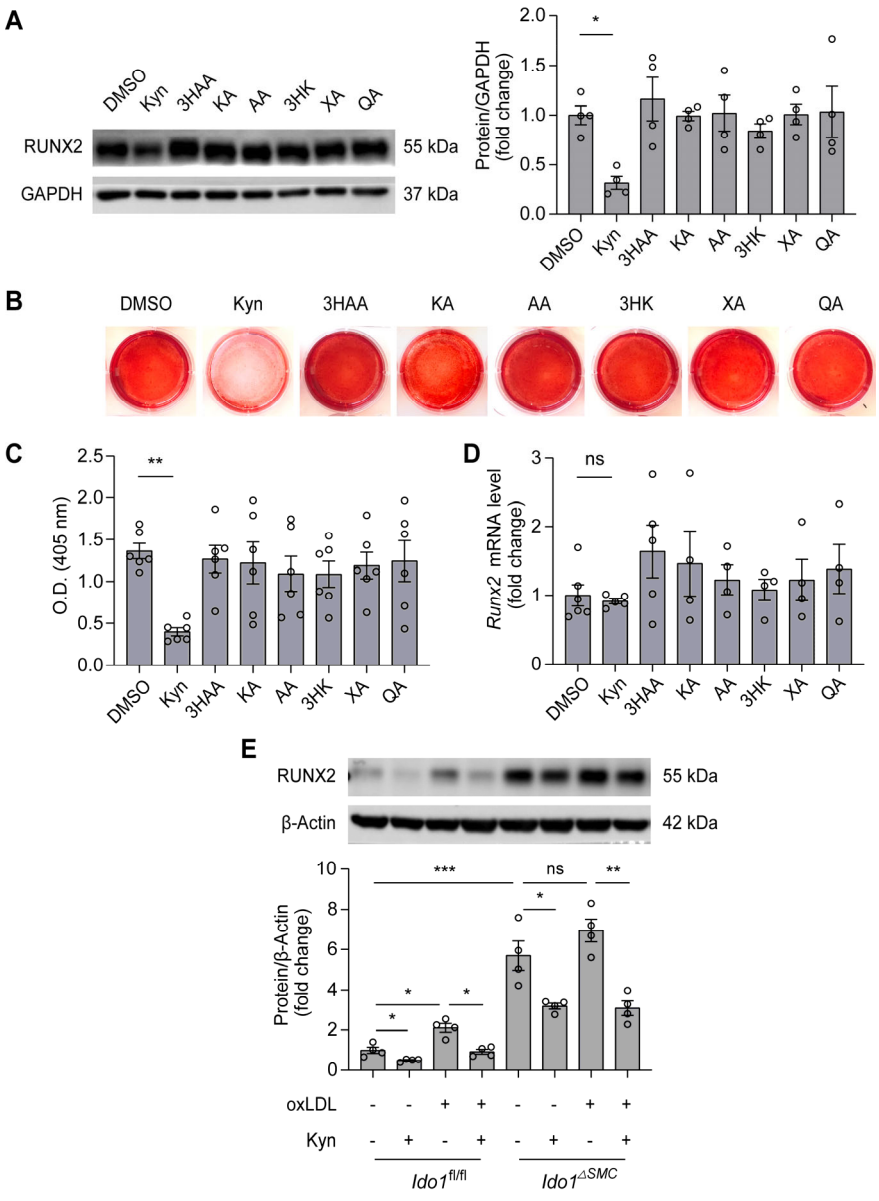
Male *Apoe*$^{-/-}$ *Ido1*$^{fl/fl}$ (n = 10), *Apoe*$^{-/-}$ *Ido1*$^{fl/fl}$ *Myh11*^{Cre} (*Apoe*$^{-/-}$ *Ido1*$^{\Delta SMC}$, n = 14), and *Apoe*$^{-/-}$ *Ido1*$^{fl/fl}$ *lysM*^{Cre} (*Apoe*$^{-/-}$ *Ido1*$^{\Delta M\Phi}$, n = 11-12) mice were fed with Western diet for 24 weeks.

A and **B**, Quantification of atherosclerotic lesion size (left panel) and percentage of lesion size (right panel) within lumen area in aortic root (**A**) and brachiocephalic artery tissue sections (**B**) in the indicated mice.

C–G, Biochemical measurement of serum levels of triglyceride (**C**), cholesterol (**D**), calcium (**E**), blood glucose (**F**), and body weight (**G**) levels in the indicated mice.

H, Kynurenine, tryptophan levels, and Kyn/Trp ratios determined in aorta lysates of indicated mice. (n = 10-14)

Results are presented as mean ± SEM, and analyzed using One-way ANOVA with Dunnett's post hoc test for **A-G**, Welch's ANOVA test with Dunnett's post hoc test for **H**. $^{**}P < 0.01$, ns indicates $P > 0.05$.



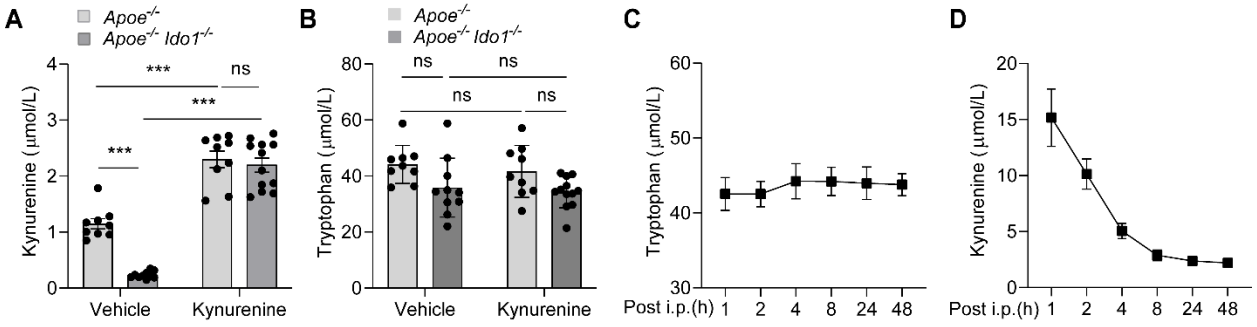
Supplemental Figure 7. Effects of IDO1 activity-related metabolites on RUNX2 expression and calcification formation in VSMCs

A, Western blot analysis of primary VSMCs isolated from *Ido1*$^{fl/fl}$ *Myh11*^{Cre} mice and cultured in osteogenic media for 14 days combined with DMSO or indicated tryptophan-derived metabolites (kynurenine, 50 μmol/L; 3HAA, 50 μmol/L; KA, 75 μmol/L; anthranilic acid (AA), 100 μmol/L; 3HK, 100 μmol/L; XA, 200 μmol/L; QA, 1 mmol/L) (n = 4).

B-D, Representative images (**B**) and quantitative results (**C**) of alizarin red staining and RT-PCR analysis of *Runx2* expression (**D**) performed in primary VSMCs with indicated treatment (n = 4-6).

E, Western blot analysis of RUNX2 expression in primary VSMCs isolated from $Ido1^{fl/fl}$ $Myh11^{Cre}$ mice with indicated treatment for 72 hours (n = 4).

Results are presented as mean ± SEM, and analyzed using one-way ANOVA with Dunnett's post hoc test for **A-D**. Two-way ANOVA with Tukey's post hoc test is used for **E**. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, ns indicates *P* > 0.05.



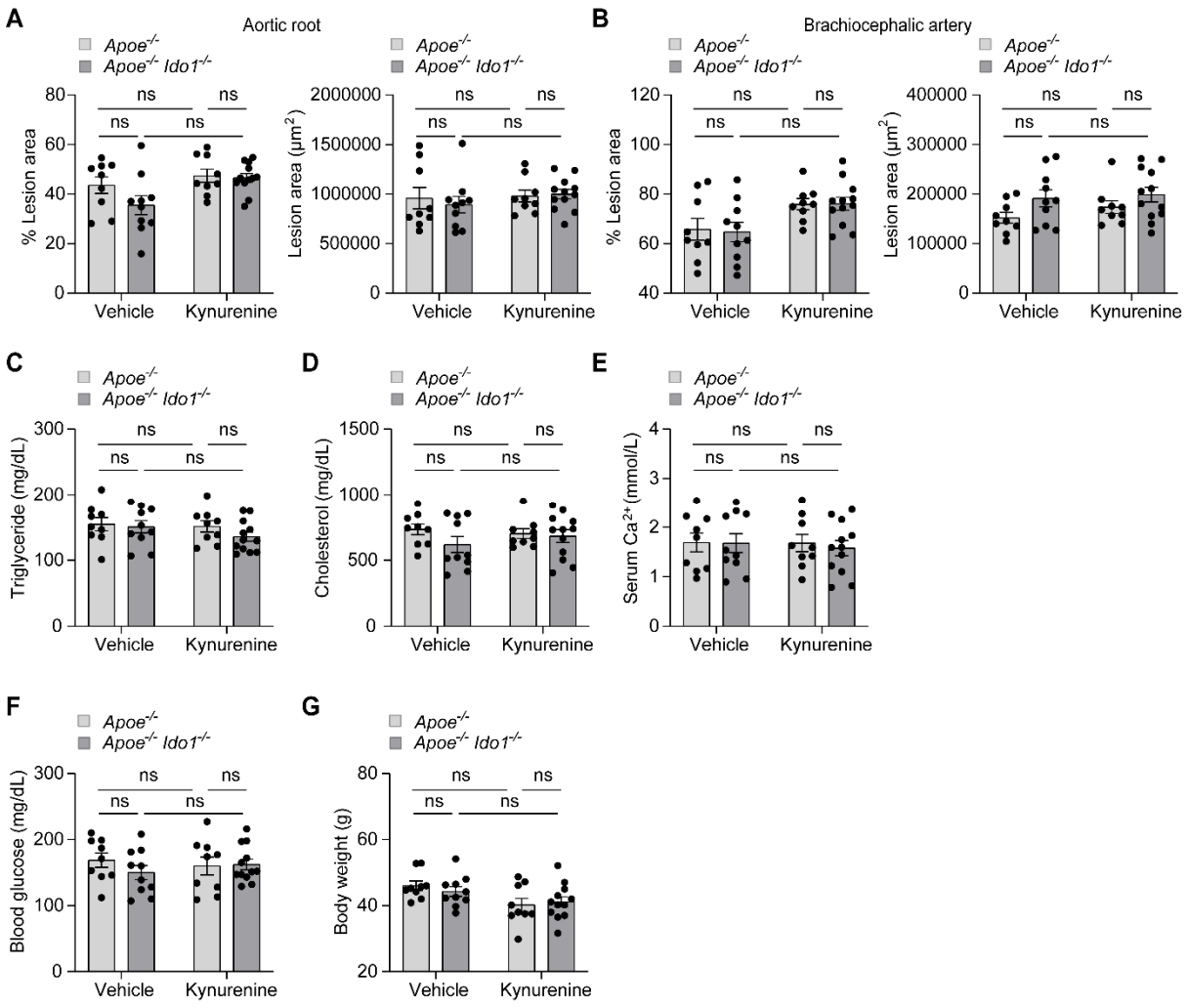
Supplemental Figure 8. Serum levels of tryptophan and kynurenine post intraperitoneal administration in vivo

Male *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$ *Ido1*$^{-/-}$ mice were fed with Western diet for 16 weeks, then supplemented with vehicle or kynurenine (100 mg/kg) via intraperitoneal injection every 48 hours together with the Western diet for another 8 weeks.

A and **B**, Kynurenine (**A**) and tryptophan (**B**) levels in serum of vehicle- or kynurenine-injected mice of the indicated genotypes 48 hours after last intraperitoneal injection (n = 9–12).

C and **D**, Serum tryptophan (**C**) and kynurenine (**D**) levels in *Apoe*$^{-/-}$ mice at indicated periods after injection with a single-dose of kynurenine (100 mg/kg) (n = 6-8).

Results are presented as mean ± SEM and analyzed using two-way ANOVA with Tukey's post hoc test in **A**. ***$P < 0.001$, ns indicates $P > 0.05$.



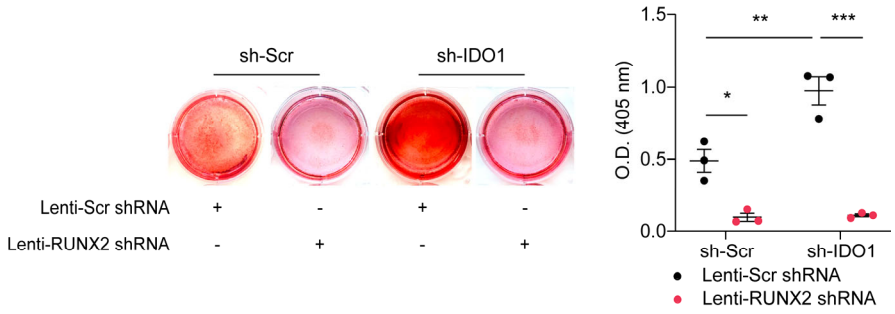
Supplemental Figure 9. Kynurenine has no effect on plaque formation in situ, or on metabolic parameters

Male *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$ *Ido1*$^{-/-}$ mice were fed with Western diet for 16 weeks, then supplemented with vehicle or kynurenine (100 mg/kg) via intraperitoneal injection every 48 hours together with the Western diet for another 8 weeks.

A and **B**, Quantification of atherosclerotic lesion size (left panel) and percentage of lesion size (right panel) within lumen area in aortic root (**A**) and brachiocephalic artery (**B**) tissue sections in indicated mice (n = 9-12).

C–G, Biochemical measurement of serum levels of triglyceride (**C**), cholesterol (**D**), calcium (**E**), blood glucose (**F**), and body weight (**G**) in the indicated mice (n = 9-12).

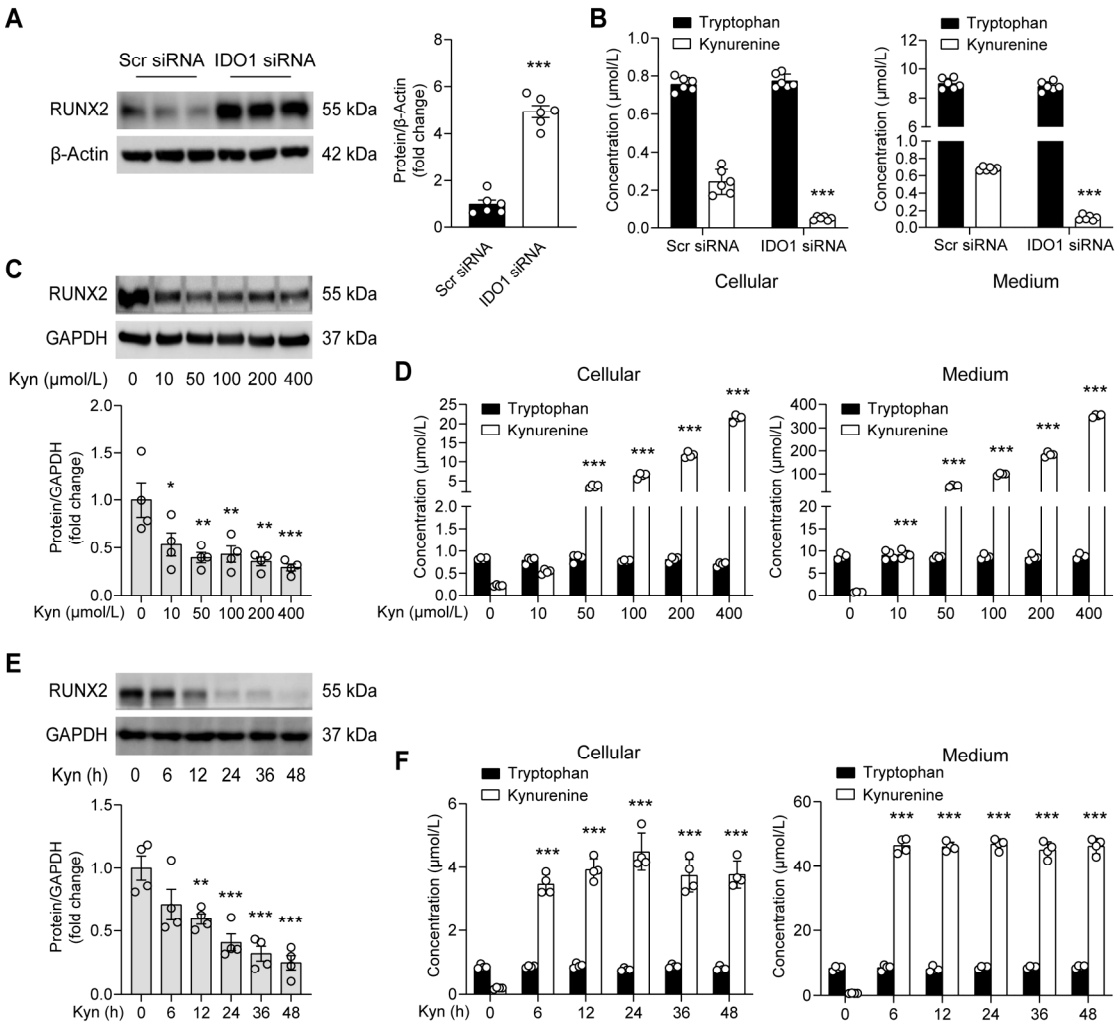
Results are presented as mean ± SEM, and analyzed using two-way ANOVA with Tukey's post hoc test. ns indicates $P > 0.05$.



Supplemental figure 10. IDO1 modulates osteogenic reprogramming of VSMCs in a RUNX2-dependent manner in human coronary VSMCs

Representative and quantitative results of alizarin red staining of human coronary VSMCs. The VSMCs were pre-transfected with lentivirus encoding scramble shRNA or IDO1 shRNA and then infected with indicated lentivirus and cultured in osteogenic media for 14 days (n = 3).

Results are presented as mean ± SEM, and analyzed using two-way ANOVA with Tukey' post hoc test. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.



Supplemental Figure 11. IDO1 regulates RUNX2 expression through kynurenine production in human coronary VSMCs

A, Western blot analysis of RUNX2 expression in human coronary VSMCs transfected with Scr siRNA or IDO1 siRNA for 48 hours (n = 6).

B, Tryptophan and kynurenine levels in lysed cells or in medium of cultured human coronary VSMCs transfected with Scr siRNA or IDO1 siRNA for 48 hours (n = 6).

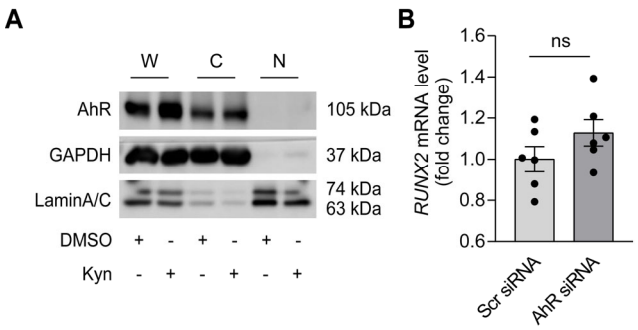
C, Western blot analysis of RUNX2 expression in human coronary VSMCs pre-transfected with RUNX2 plasmid for 24 hours and then treated with kynurenine at indicated dosage (n = 4).

D, Levels of tryptophan and kynurenine in lysed cells (left panel) or in medium (right panel) of human coronary VSMCs pre-transfected with RUNX2 plasmid for 24 hours and then treated with kynurenine at indicated dosages (n = 4).

E, Western blot analysis of RUNX2 expression levels in human coronary VSMCs pre-transfected with RUNX2 plasmid for 24 hours, followed by treatment with kynurenine (50 μmol/L) for the indicated periods (n = 4).

F, Tryptophan and kynurenine levels in lysed cells or medium of cultured human coronary VSMCs pre-transfected with RUNX2 plasmid for 24 hours, followed by treatment with kynurenine (50 μmol/L) for the indicated periods (n = 4).

Results are presented as mean ± SEM. *P* values are assessed using two-tailed unpaired Student's *t*-tests for **A** and **B**. One-way ANOVA with Dunnett's post hoc test is used for **C**-**F**. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 *vs* Control group (Scr siRNA, Kyn 0μmol/L, or Kyn 0h).

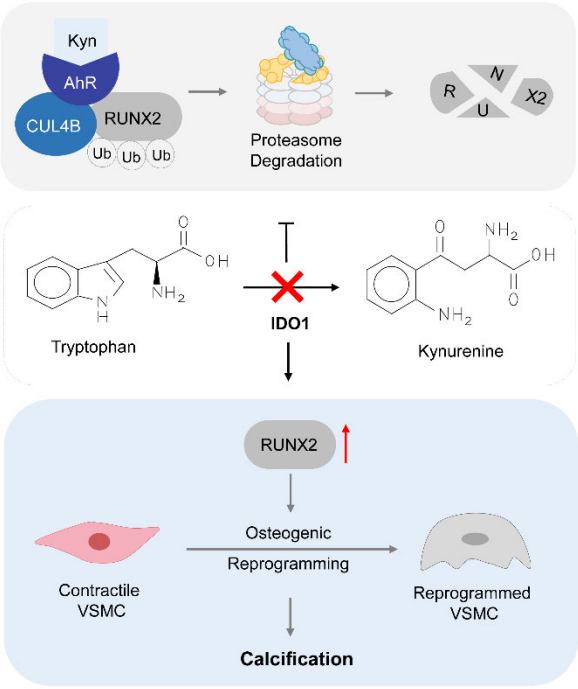


Supplemental Figure 12. RUNX2 mRNA expression is not affected by kynurenine or AhR

A, Western blot analysis of AhR expression in fragmental lysates of human coronary VSMCs treated with DMSO or kynurenine (50 μmol/L) for 48 hours (n = 3). W: whole cell lysate. C: cytosolic lysate. N: nuclear lysate.

B, Quantitative RT-PCR analysis of *RUNX2* expression in human coronary VSMCs transfected with Scr siRNA or AhR siRNA for 48 hours (n = 6).

Results are presented as mean ± SEM. *P* values are assessed using two-tailed unpaired Student's *t*-test. ns indicates $P > 0.05$.



Supplemental Figure 13. Graphical scheme of IDO1-catabolized kynurenine alleviating VSMCs osteogenic reprogramming and calcification by RUNX2 downregulation