SUPPLEMENTAL MATERIAL

Therapeutic antibody against phosphorylcholine preserves coronary function and attenuates vascular ^{18}F-FDG uptake in atherosclerotic mice

Mia Ståhle, MSc,[a] Johanna M. U. Silvola, PhD,[a] Sanna Hellberg, PhD,[a] Margreet de Vries, PhD,[b] Paul H. A. Quax, PhD,[b] Jeffrey Kroon, PhD,[c] Petteri Rinne, PhD[d,e] Alwin de Jong, MSc,[b] Heidi Liljenbäck, MSc,[a,e] Nina Savisto, PhD,[a] Anna Wickman, PhD,[f] Erik S. G. Stroes, MD, PhD,[c,g] Seppo Ylä-Herttuala, MD, PhD,[h] Pekka Saukko, MD, PhD,[i] Tommy Abrahamsson, MD, PhD,[j] Knut Pettersson, PhD,[j] Juhani Knuuti, MD, PhD,[a,k] Anne Roivainen, PhD,[a,e] Antti Saraste, MD, PhD[a,k,l,m]

[a]Turku PET Centre, University of Turku, Turku, Finland, [b]Einthoven Laboratory for Experimental Vascular Medicine, Department of Surgery, Leiden University Medical Center, Leiden, the Netherlands, [c]Department of Experimental Vascular Medicine, Amsterdam Cardiovascular Sciences, Amsterdam UMC, University of Amsterdam, Amsterdam, the Netherlands, [d]Research Center for Integrative Physiology and Pharmacology, Institute of Biomedicine, University of Turku, Turku, Finland [e]Turku Center for Disease Modeling, University of Turku, Turku, Finland, [f]i3tex AB, Gothenburg, Sweden, [g]Department of Vascular Medicine, Academic Medical Center, Amsterdam UMC, Amsterdam, the Netherlands, [h]A.I. Virtanen Institute for Molecular Sciences, University of Eastern Finland, Kuopio, Finland, [i]Department of Pathology and Forensic Medicine, University of Turku, Turku, Finland, [j]Athera Biotechnologies AB, Stockholm, Sweden, [k]Turku PET Centre, Turku University Hospital, Turku, Finland, [l]Heart Center, Turku University Hospital, Turku, Finland, [m]Institute of Clinical Medicine, Turku University Hospital, Turku, Finland

Address for Correspondence: Antti Saraste**,** Turku PET Centre, Turku University Hospital, Kiinamyllynkatu 4-8, FI-20520 Turku, Finland. Tel: +358 2 3130083, Fax: +358 2 2318191, E-mail: antti.saraste@utu.fi

Supplemental methods

Blood samples

Glucose levels were measured with a glucometer (Bayer Contour, Bayer AG, Leverkusen, Germany) from whole blood samples withdrawn from the femoral vein at the time of ^{18}F-FDG injection. Plasma concentrations of cholesterol, low-density lipoprotein (LDL), high-density lipoprotein (HDL), and triglycerides were measured using a Konelab 20i chemistry analyzer (Thermo Fisher Scientific, MA, USA), while X19-mu antibody was measured from terminal blood samples collected by heart puncture. X19-mu and PC-mAb were measured with a modified CVDefine ELISA kit (Athera Biotechnologies AB, Stockholm, Sweden) where the original secondary antibody (detecting human IgM) was replaced with anti-IgG (AffiniPure goat anti-mouse IgG [H+L] and goat anti-human IgG [H+L], respectively; Jackson Immuno Research Laboratories, Baltimore, MD, USA). The assay is based on PCs covalently linked to bovine serum albumin coated onto 96-well microtiter plates. All incubations were carried out at room temperature.

Histology and immunofluorescence

Primary antibodies and detection methods are shown in Supplemental Table 1. The sections were scanned with a digital slide scanner (Pannoramic 250 Flash; 3DHistech Ltd., Budapest, Hungary) for histology and with a fluorescence scanner (Pannoramic MIDI) or microscopes (Nikon Ti-2E, objective 20x/0.75; Nikon, Amsterdam, the Netherlands, and Zeiss LSM700, objective 40x/1.4 Oil DIC; Zeiss GmbH, Oberkochen, Germany) for immunofluorescence. One to three sections per staining per mouse were analyzed using Image-J 1.46 software (NIH, Bethesda, MD, USA). The percentage of intimal area positive for macrophages, IL-1β and MCP-1 staining was calculated, and lesion endothelial lining positive for VCAM-1 and ICAM-1 was graded as 1 (<30%), 2 (30-60%) or 3 (>60%). Apoptotic macrophages were measured as the number of Mac-3 macrophages positive for caspase-3 per intimal area (mm^2).

Endothelium-mediated vasodilatation

Endothelium-mediated vasodilatation was investigated in male apolipoprotein E deficient ($ApoE^{-/-}$) mice (B6.129P2-$Apoe^{tm1Unc}$/J, Taconic, Silkeborg, Denmark) after feeding with a high-fat diet for 20 weeks, starting at the age of 8 weeks as previously described (2). The mice were randomized to receive intraperitoneal injections containing either 0.9% saline solution as a vehicle (n=8) or 10 mg/kg X19-mu antibody (n=7), once a week, for 8 weeks. Then, mice were anesthetized with isoflurane and arterial blood pressure was monitored for 3 minutes after intravenous injection of an equal volume of either saline or methacholine (3 μg/kg of Acetyl-β-methylcholine-chloride, 98%, Sigma-Aldrich, St. Louis, MO, USA) using a 1250 Hz catheter-tip pressure transducer (Samba Sensor preclin 420LP, Gothenburg, Sweden) introduced into the aortic arch through a left carotid artery incision. The results were expressed as the maximal change in systolic and diastolic blood pressure (SBP and DBP) caused by methacholine subtracted the change obtained by saline injection.

Cell culture

Primary human aortic endothelial cells (HAECs) were purchased from Lonza (Baltimore, MD, USA). Cells were seeded on fibronectin-coated tissue culture-treated wells. HAECs were maintained in EGM-2 medium (Lonza) at 37 and 5% CO2). Cells between passage 4-7 were used for experiments.

Lipoprotein(a) [Lp(a)] isolation

Lipoprotein fractions were isolated from plasma of three different male and female normolipidemic volunteers (both male and female from different ages). A detailed procedure of the used isolation method can be found elsewhere (1). While in this manuscript the Lp(a) fraction, instead of the LDL fraction was isolated. In short, samples were centrifuged under vacuum conditions for 19 hours at 29.000 rpm at 10degr in a SW 41 Ti rotor without brake

(Beckman Coultier Inc., CA, USA). After the run, the obtained Lp(a) fraction was sliced out, dialyzed and filter sterilized (0.2 µm pore size; Sartorius, Göttingen, Germany). Subsequently, Lp(a) was concentrated using Amicon centrifugal filter units (10.000 MWCO; Millipore). After measuring Lp(a) concentrations using commercially available immunoturbidimetric enzymatic asays (Diasys, Holzheim, Germany) on a Selectra sysyem (Sopachem, Ochten, The Netherlands). Finally, Lp(a) was used as indicated.

Nitric oxide (NO) assay kit

Intracellular NO was measured using a commercially available Nitric Oxide Assay Kit (fluorometric) according manufacturers' instructions (ab65327, Abcam, Cambridge, UK). Since NO is rapidly converted to nitrite and nitrate, the total concentration of nitrite and nitrate is used as a quantitative measure of NO production. In short, after stimulation, cells were washed twice with cold PBS and resuspended in assay buffer on ice. Next, cells were homogenized, centrifuged and assayed. Fist, nitrate is converted to nitrite by nitrate reductase. Subsequently, nitrate reacts with the fluorescent DAN (2,3 diaminoaoththalene), where the intensity is proportional to the total nitric oxide production. Fluorescent intensity was measured using a FLUOstar Galaxy (BMG Labtechnologies, Offenburg, Germany) at Ex/Em = 460/450 nm and a standard curve was used to calculate the concentrations.

Cytokine measurements

Cytokine (protein) production was measured in supernatants of HAECs using commercial enzyme-linked immunosorbent assay kits for IL-6 and IL-8 (ThermoFisher), following the manufacturers' instructions

RNA isolation and quantitative PCR analysis

RNA was extracted from cultured cells using Trizol and cDNA was synthesized with iScript (Biorad, Veenendaal, The Netherlands). qPCR was performed with 500 ng cDNA using SYBR

Green Fast on a ViiAa7 PCR machine (Applied Biosystems, Bleiswijk, The Netherlands). Primers for *IL6, IL8, ICAM1, VCAM1* and *36B4* were obtained from Sigma Aldrich (Zwijndrecht, The Netherlands). Gene expression was normalized to the housekeeping gene *36B4*. The primer sequences that were used are: *IL6* Forward: CTG CAG AAA AAG GCA AAG AAT CTA, Reverse: GTT GTC ATG TCC TGC AGC C*; IL8* Forward: TGT TCC ACT GTG CCT TGG TTT CTC C, Reverse: TGC TTC CAC ATG TCC TCA CAA CAT CAC; *ICAM1* forward: ATG GCA ACG ACT CCT TCT CG, reverse: GCC GGA AAG CTG TAG ATG GT; *VCAM1* forward: TGT CAA TGT TGC CCC CAG A, reverse: TGC TCC ACA GGA TTT TCG GA; *36B4* forward: GAA CAT CTC CCC CTT CTC CTT C, reverse: ATT GCG GAC ACC CTC TAG GAA.

Biodistribution of ^{18}F-FDG uptake

Samples from other organs including the brain, heart, intestine, kidney, liver, lung, lymph node, muscle, pancreas, spleen, and white and brown adipose tissue were prepared and total radioactivity was measured using a gamma counter (Triathler 3", Hidex Oy, Turku, Finland). Radioactivity values were normalized for injected radioactivity dose per animal weight, decay, and the weight of the tissue sample. Results were expressed as standardized uptake values (SUV).

Supplemental results

X19-mu treatment improved endothelium-mediated vasodilatation

Blood pressure was similar in vehicle- and X19-mu-treated mice before methacholine injection (SBP 139±21 vs. 135±16 mmHg, p=0.70 and DBP 88±10 mmHg vs. 88±15 mmHg, p=0.99, respectively). Methacholine had no effect on arterial blood pressure in vehicle-treated mice, whereas there was a transient reduction in blood pressure without change in heart rate in X19-mu-treated mice (SBP +1.9±4.9 mmHg vs. -5.0±5.6 mmHg, p=0.036 vs. vehicle, and DBP +0.50±3.0 mmHg vs. -4.3±4.0 mmHg, p=0.023 vs. vehicle). (Supplemental Figure 3)

Supplemental Tables

Supplemental Table 1. Primary antibodies and detection methods used for immunofluorescence and immunohistochemical stainings.

Antibody	Clone	Dilution	Manufacturer	Detection
Mac-3 *	Monoclonal rat anti-mouse Mac-3, 550292	IF 1:200 IHC 1:5000	BD Pharmingen, Franklin Lakes, NJ, US	IF: Donkey anti-rat Alexa 594, A21209, 1:250 (Invitrogen, Carlsbad, CA, US) IHC: Vectastain ABC-HRP kit (Vector Laboratories, Burlingame, CA, USA) + chromogen (DAB, Dako K3468)
CCR2	Polyclonal rabbit anti-mouse CCR2, GR172538	1:400	Abcam, Cambridge, UK	Donkey anti-rabbit Alexa 488, A21206, 1:250 (Invitrogen, Carlsbad, CA, US)
CD206	Polyclonal rabbit anti-mouse CD206, GR216007-1	1:300	Abcam, Cambridge, UK	Donkey anti-rabbit Alexa 488, A21206, 1:250 (Invitrogen, Carlsbad, CA, US)
VCAM-1	Monoclonal rabbit anti-mouse VCAM-1, ab134047	1:100	Abcam, Cambridge, UK	Donkey anti-rabbit Alexa 594, A21207, 1:200 (Invitrogen, Carlsbad, CA, US)
ICAM-1	Polyclonal rabbit anti-mouse ICAM-1, sc-1511-R	1:300	SantaCruz, Dallas, TX, US	Goat anti-rabbit Alexa 647, A21244, 1:1000 (Invitrogen, Carlsbad, CA, US)
IL-1β	Polyclonal rabbit anti-mouse IL-1β, ab9722	1:100	Abcam, Cambridge, UK	Donkey anti-rabbit Alexa 594, A21207, 1:200 (Invitrogen, Carlsbad, CA, US)
MCP-1	Monoclonal rat anti-mouse CCL2/MCP-1, NBP1-42280	1:300	Novus Biologicals, Abingdon, UK	Goat anti-rat Alexa 647, A21247, 1:1000 (Invitrogen, Carlsbad, CA, US)
Caspase-3 †	Polyclonal rabbit anti-mouse Cleaved Caspase-3, 9661-s	1:400	Cell Signaling Technology, Leiden, The Netherlands	Goat anti-rabbit Alexa 555, A21428, 1:1000 (Invitrogen, Carlsbad, CA, US)
CD31	Polyclonal rabbit anti-mouse, CD31/PECAM-1, sc-1506-R	1:100	SantaCruz, Dallas, TX, US	Donkey anti-rabbit Alexa 488, A21206, 1:250 (Invitrogen, Carlsbad, CA, US)
PC ‡	Non-commercial monoclonal fully human PC-mAb	1:10	Athera Biotechnologies AB, Stockholm, Sweden	Primary antibody is labeled with Cy5 Mounting medium: ProLong TM Gold antifade reagent with DAPI, P36935 (Invitrogen, Carlsbad, CA, US)

	Isotype controls		
Mac-3	Rat IgG, E019346	Thermo Fischer Scientific, Waltham, MA, US	Isotype controls diluted to match primary antibodies, detection methods as above.
CCR2, CD206, ICAM-1, CD31, Caspase-3	Rabbit polyclonal IgG, CTL-4112 (910801)	BioLegend, San Diego, CA, US	
VCAM-1, IL-1β	Rabbit IgG, 31235	Thermo Fischer Scientific, Waltham, MA, US	
MCP-1	Rat IgG, 14-4031-81	Thermo Fischer Scientific, Waltham, MA, US	
PC	Goat IgG, sc-2028	SantaCruz, Dallas, TX, US	

*Co-staining with CCR2 or CD206 antibody, † Co-staining with Mac-3 antibody, ‡ Co-staining with Mac-3 and CD31 antibodies.
IF; immunofluorescence, IHC; immunohistochemistry.

Supplemental Table 2. Coronary flow velocity and reserve before and after 6 week treatment with vehicle or X19-mu.

	Rest			Adenosine			CFR		
	Week 0	Week 6	*p-value*†	Week 0	Week 6	*p-value*†	Week 0	Week 6	*p-value*†
Mean diastolic flow velocity (mm/s)									
Vehicle	240 ± 87	270 ± 110	0.40	450 ± 160	370 ± 160	0.29	1.9 ± 0.30	1.4 ± 0.23	0.006
X19-mu	230 ± 73	230 ± 110	0.94	370 ± 140	380 ± 180	0.82	1.6 ± 0.24	1.7 ± 0.24	0.32
*p-value**	0.73	0.37		0.22	0.90		0.024	0.012	

Values are mean ± SD (n=10/group). *Student's *t*-test for unpaired and †for paired measurements.

Supplemental Table 3. Biodistribution of ^{18}F-FDG uptake in LDLR$^{-/-}$ApoB$^{100/100}$ mice at the time of randomization (week 0) and after a 6 week treatment with vehicle or X19-mu antibody.

	Week 0 (n=11)	**Vehicle** (n=16)	**X19-mu** (n=16)
BAT	1.0 ± 0.39	1.4 ± 0.29*	1.4 ± 0.26†
Blood	0.25 ± 0.045	0.24 ± 0.047	0.23 ± 0.082
Brain	1.3 ± 0.47	1.6 ± 0.33	1.3 ± 0.31‡
Heart	25 ± 6.2	23 ± 7.6	23 ± 7.5
Intestine	1.9 ± 0.73	1.9 ± 0.59	1.8 ± 0.56
Kidney	1.5 ± 0.70	1.5 ± 0.54	1.3 ± 0.38
Liver	0.81 ± 0.33	0.62 ± 0.071	0.62 ± 0.24
Lung	2.2 ± 0.50	2.1 ± 0.43	1.9 ± 0.36
Lymph node	1.7 ± 0.58	1.8 ± 0.36	1.8 ± 0.31
Muscle	0.69 ± 0.30	0.35 ± 0.18†	0.48 ± 0.33
Pancreas	0.68 ± 0.18	0.89 ± 0.24*	0.86 ± 0.23
Spleen	2.9 ± 0.60	2.6 ± 0.41	2.4 ± 0.42†
WAT	0.079 ± 0.054	0.17 ± 0.12	0.19 ± 0.11*

Results are expressed as standardized uptake values (SUV mean ± SD);
ANOVA followed by Dunnett's post-hoc test for the week 0 group and
Student's *t*-test for unpaired (vehicle vs. X19-mu) measurements;
*p<0.05, †p<0.01 vs. week 0 and ‡p<0.05 vehicle vs. X19-mu.
BAT; brown adipose tissue, WAT; white adipose tissue.

Supplemental Figure legends

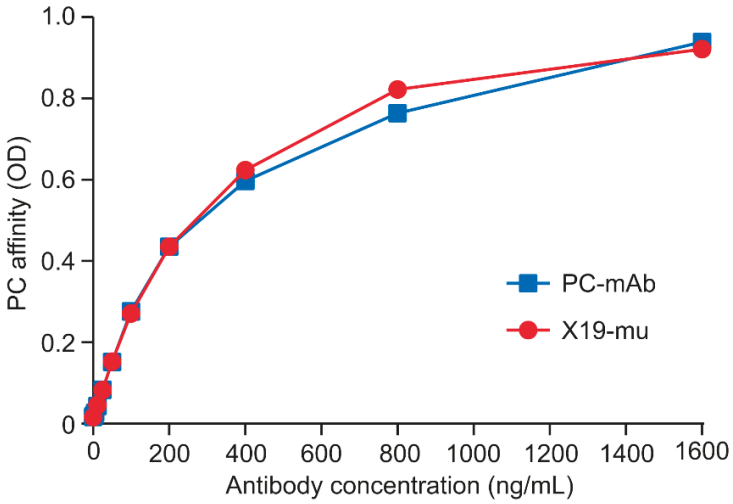
Supplemental Figure 1. Binding affinities of a fully human PC-mAb and X19-mu antibodies to phosphorylcholine (PC) are comparable. The binding affinity curves measured by ELISA (expressed as optical density OD) show a similar binding efficacy to PC between PC-mAb and X19-mu antibody. Curves represent a mean of three antibody batches analyzed for both PC-mAb and X19-mu.

Supplemental Figure 2. Control immunofluorescence stainings with non-immune IgGs. Images of atherosclerotic lesions from the aortic root stained with appropriate isotype controls and secondary antibodies. Rabbit polyclonal IgG for CCR2 and CD206 antibodies (scale bar = 50 μm) (**A**), rabbit IgG for VCAM-1, IL-1β and ICAM-1, rat IgG for MCP-1 and rabbit IgG for Caspase-3 antibody (scale bar = 50 μm) (**B**), and rabbit polyclonal IgG for CD31, goat IgG for PC and rat IgG for Mac-3 antibody (scale bar = 75 μm) (**C**),

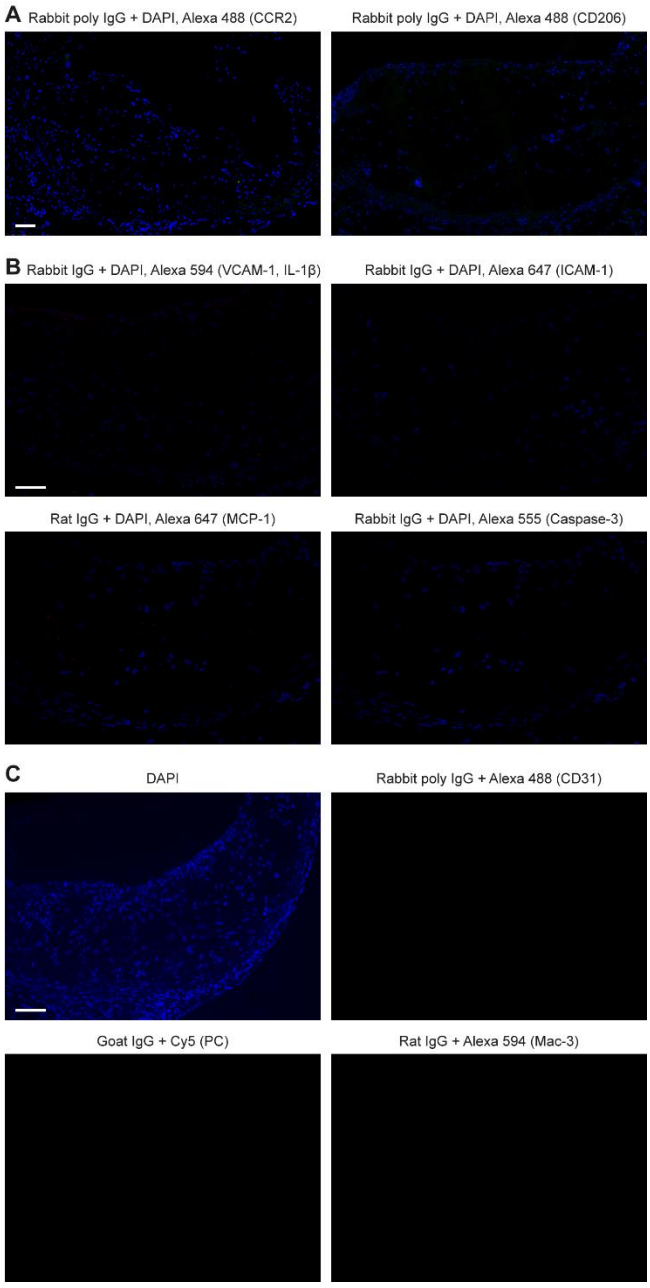
Supplemental Figure 3. X19-mu treatment improves endothelium-mediated vasodilatation. Representative images of the arterial blood pressure in response to methacholine (MCh) over time recorded by an aortic catheter in a mouse treated with X19-mu or vehicle (**A**). Treatment with X19-mu caused a transient reduction in blood pressure in response to MCh compared with vehicle treatment (**B**). Maximal change in blood pressure shown as individual data points with mean±SD; Student's *t*-test for unpaired measurements; n=6-8/group. DBP=diastolic blood pressure, SBP=systolic blood pressure.

Supplemental Figure 4. X19-mu treatment reduces ^{18}F-FDG uptake in atherosclerotic lesions both in female and male mice. ^{18}F-FDG uptake in atherosclerotic lesions (normalized by the activity of lesion-free vessel wall) was lower after a 6 week treatment with X19-mu in both females and males compared with vehicle. Results are expressed as individual data points with mean ± SD; Student's *t*-test for unpaired measurements; n=8/group.

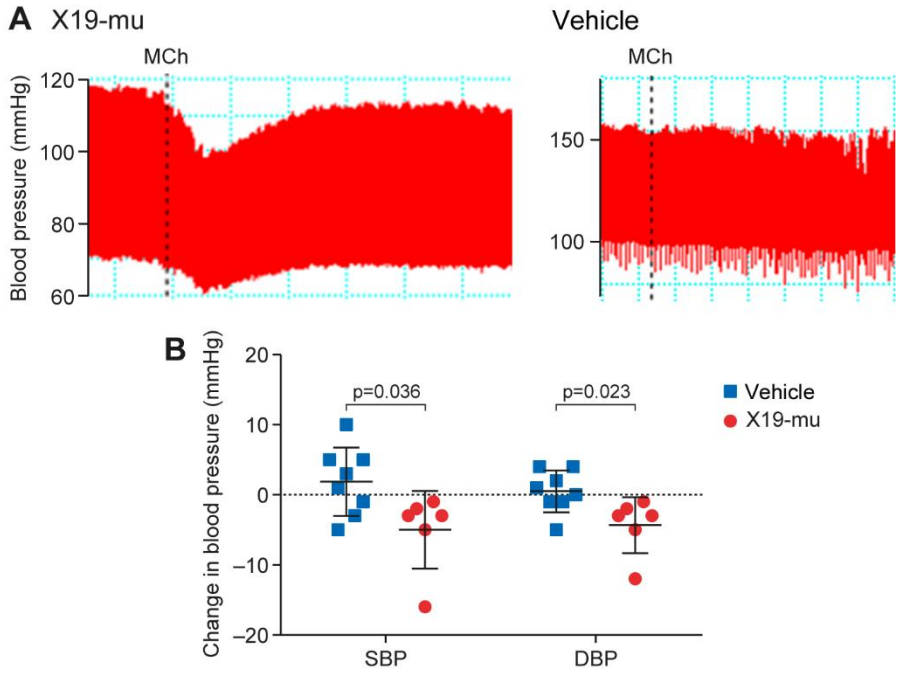
Supplemental Figures



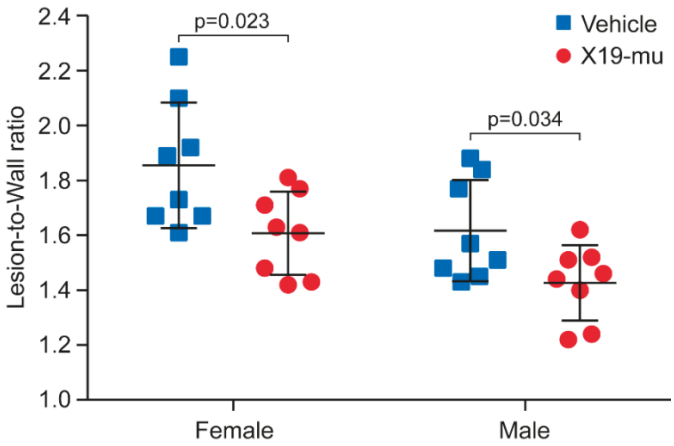
Supplemental Figure 1.



Supplemental Figure 2.



Supplemental Figure 3.



Supplemental Figure 4.

References

1 Schnitzler J, Bernelot Moens J, Tiessens F, et al. Nile Red Quantifier: a novel and quantitative tool to study lipid accumulation in patient-derived circulating monocytes using confocal microscopy. J Lipid Res 2017;58:2210–19.

2. Ewing MM, de Vries MR, Nordzell M, et al. Annexin A5 therapy attenuates vascular inflammation and remodeling and improves endothelial function in mice. Arterioscler Thromb Vasc Biol 2011;31:95–101.