

ORIGINAL RESEARCH

# Kallikrein-1 Blockade Inhibits Aortic Expansion in a Mouse Model and Reduces Prostaglandin E<sub>2</sub> Secretion From Human Aortic Aneurysm Explants

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**BACKGROUND:** Abdominal aortic aneurysm (AAA) is an important cause of mortality in older adults. The kinin B2 receptor agonist, bradykinin, has been implicated in AAA pathogenesis through promoting inflammation. Bradykinin is generated from high- and low-molecular-weight kininogen by the serine protease kallikrein-1. The aims of this study were first to examine the effect of neutralizing kallikrein-1 on AAA development in a mouse model and second to test how blocking kallikrein-1 affected cyclooxygenase-2 and prostaglandin E<sub>2</sub> in human AAA explants.

**METHODS AND RESULTS:** Neutralization of kallikrein-1 in apolipoprotein E-deficient (*ApoE*<sup>-/-</sup>) mice via administration of a blocking antibody inhibited suprarenal aorta expansion in response to angiotensin (Ang) II infusion. Kallikrein-1 neutralization decreased suprarenal aorta concentrations of bradykinin and prostaglandin E<sub>2</sub> and reduced cyclooxygenase-2 activity. Kallikrein-1 neutralization also decreased protein kinase B and extracellular signal-regulated kinase 1/2 phosphorylation and reduced levels of active matrix metalloproteinase 2 and matrix metalloproteinase 9. Kallikrein-1 blocking antibody reduced levels of cyclooxygenase-2 and secretion of prostaglandin E<sub>2</sub> and active matrix metalloproteinase 2 and matrix metalloproteinase 9 from human AAA explants and vascular smooth muscle cells exposed to activated neutrophils.

**CONCLUSIONS:** These findings suggest that kallikrein-1 neutralization could be a treatment target for AAA.

**Key Words:** aneurysm ■ cyclooxygenase-2 ■ kallikrein-1 ■ prostaglandin E<sub>2</sub>

Worldwide, abdominal aortic aneurysm (AAA) is estimated to be responsible for ≈200 000 deaths each year because of aortic rupture or complications of surgical repair.<sup>1</sup> Most AAAs are identified when they are small and at low risk of rupture.<sup>2</sup> The natural history of most small AAAs is expansion to a size when surgical repair is required to prevent rupture. There is great current interest in identifying targets for the development of drug therapies that can effectively limit AAA growth and rupture.<sup>3–6</sup>

Kinins are potent bioactive peptides key in regulating vascular permeability and inflammation following tissue

injury.<sup>7</sup> It has been previously reported that administration of a kinin-receptor agonist amplified the response to angiotensin II (AngII) and markedly promoted aortic rupture and severity of aortic dilatation within a mouse model of AAA.<sup>8</sup> A kinin-receptor antagonist limited aneurysm growth and reduced aortic rupture incidence in the same experimental model.<sup>8</sup> These findings suggested that blocking kinin signaling could be a potential treatment strategy to limit AAA progression, although how to achieve this most effectively and safely remains unclear.

Within tissues, such as blood vessels, the action of kallikrein-1 (tissue kallikrein) releases the endogenous

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## CLINICAL PERSPECTIVE

### What Is New?

- Neutralization of kallikrein-1 inhibited experimental aortic aneurysm formation in a mouse model through downregulating cyclooxygenase-2 and matrix metalloproteinase 2 and 9.
- In vitro neutralization of kallikrein-1 reduced levels of cyclooxygenase-2 and secretion of prostaglandin E<sub>2</sub> and active matrix metalloproteinase 2 and 9 from human AAA explants.

### What Are the Clinical Implications?

- Kallikrein-1 may be a novel target for a drug therapy for aortic aneurysm.

## Nonstandard Abbreviations and Acronyms

<b>AKT</b>	protein kinase B
<b>AngII</b>	angiotensin II
<b>AoSMC</b>	aortic smooth muscle cell
<b>ApoE<sup>-/-</sup></b>	apolipoprotein E-deficient mouse
<b>MMP</b>	matrix metalloproteinase
<b>PGE2</b>	prostaglandin E2
<b>SRA</b>	suprarenal aorta

kinin B2 receptor ligand bradykinin from either high- or low-molecular-weight kininogen.<sup>9,10</sup> Kallikrein-1 blockade could therefore be a feasible and potentially safe way to limit kinin receptor signaling and progression of AAA pathological processes.

The aim of this study was to test whether kallikrein-1 neutralizing antibodies limited AAA progression within the AngII-infused apolipoprotein E-deficient (*ApoE<sup>-/-</sup>*) mouse model. A secondary aim was to study whether kallikrein-1 neutralizing antibodies limited release of pro-inflammatory cytokines and matrix remodeling enzyme from human AAA explant and vascular smooth muscle cells in vitro.

## METHODS

The data that support the findings of this study are available from the corresponding author upon reasonable request.

### Kallikrein-1 Inhibitors

Neutralizing antibodies to mouse (M40-A03) and human (DX-2300) kallikrein-1 and corresponding IgG controls were kindly provided by Dr Daniel Sexton (Shire, United States; formerly of Dyax Corporation).

## Mouse Model and In Vivo Study

Mouse studies were performed in accordance with institutional and ethical guidelines of James Cook University, Australia (Approval A1455) and conformed to the Australian code for the care and use of animals for scientific purposes (8th Edition, 2013), the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health, USA) and the ARRIVE criteria set by The National Centre for the Replacement, Refinement and Reduction of Animals in Research (London, UK). All experimental procedures were conducted within the Small Animal Handling Facility, James Cook University, Australia.

### Mice

Male apolipoprotein E-deficient mice (*ApoE<sup>-/-</sup>*) were purchased from The Animal Resources Centre, Western Australia and housed under a 12:12-hour light–dark cycle (relative humidity: 55%–60%; temperature: 22±1°C) in an individually Ventilated Cage unit (Tecniplast). Standard chow and water were provided ad libitum. Male mice were used because of the greater propensity for AngII-induced AAA.

### Aortic Aneurysm Model

Aortic dilatation was induced in 6-month-old male *ApoE<sup>-/-</sup>* mice by subcutaneous infusion of AngII (1.0 µg/kg per minute) for 28 days as previously described.<sup>8,11,12</sup> Briefly, an osmotic micro-pump (ALZET Model 1004, Durect Corporation) containing AngII (#A9525, Merck) dissolved in sterile water was inserted into the subcutaneous space, left of the dorsal midline under general anaesthesia (4% isoflurane) to administer AngII at a rate of 1.0 µg/kg per minute over 28 days.

### Intervention

Mice were randomly allocated to intraperitoneal administration of M40-A03 (10 mg/kg; n=15) or isotype control (n=15) commenced 24 hours before placement of the AngII infusion pumps, then every 3 days over the 28-day AngII infusion period. Sudden mouse fatality required necropsy within 24 hours to confirm aortic rupture as the cause of death. Mice completing the study protocol were isoflurane-sedated before CO<sub>2</sub>-induced euthanasia. Harvested aortas were PBS-perfused and digitally photographed (Coolpix 4500, Nikon).

### Assessment of Suprarenal Aortic Dilatation

All outcome assessment was performed by an observer blinded to group allocation. Aortic diameter was the primary outcome of the mouse experiment.

Ultrasound measurements of the suprarenal aorta (SRA; ie, aortic hiatus to the left renal artery) were obtained before AngII infusion (day 0) and then at days 14 and 28. Scans were performed in sedated mice (4% isoflurane) using a MyLab™ 70 VETXV platform (Esaote, Italy) with a 40-mm linear transducer at an operating frequency of 10 MHz (LA435; Esaote, Italy) to provide a sagittal image of the SRA. Maximum outer-to-outer-wall orthogonal SRA diameter was measured at peak systole using the caliper measurement feature. Interobserver reproducibility of ultrasound analysis in our laboratory is excellent.<sup>13</sup>

## Cell Culture

### Preparation of Neutrophil-Derived Conditioned Media

Neutrophils from human-donor whole peripheral blood were purified by dextran T500 (551005009006, Pharmacosmos) centrifugation followed by Ficoll-Hypaque (1077, Merck) gradient centrifugation and hypotonic lysis of residual erythrocytes by red blood cell lysis buffer (00-4333-57, e-Bioscience). Cells were washed and resuspended with Hanks balanced salt solution (14025092, ThermoFisher Scientific) and used immediately for experiments. Trypan blue exclusion demonstrated 98% cell viability. Purified neutrophils ( $1.2 \times 10^6$ /mL) were incubated in the presence of either Hanks balanced salt solution (control), AngII (10 nmol/L; termed CM1), or AngII+kallikrein-1 neutralizing antibody (DX-2300; 5  $\mu$ g/mL; termed CM2) for 30 minutes at 37°C, 5% CO<sub>2</sub>, with the resulting conditioned culture supernatant assayed for markers of neutrophil activation.

### Vascular Smooth Muscle Cell Studies

Human aortic vascular smooth muscle cells (AoSMC; Lonza) were maintained in DMEM+10% fetal bovine serum at 37°C, 5% CO<sub>2</sub>, at a density of  $2 \times 10^5$  to  $1 \times 10^6$  cells/mL (passage 5–8). Experimental AoSMC seeded at  $5 \times 10^4$  cells/mL were rendered quiescent in serum-free medium (18 hours) before incubation under the following conditions: (1) control media; (2) control media+10% volume per volume (vol/vol) CM1; and (3) control media+10% vol/vol CM2. Cells harvested after 24 hours were prepared for protein and gene expression analyses.

### Human AAA Biopsies

Informed consent from male patients undergoing elective repair of 5- to 6-cm aneurysm allowed collection of full-thickness biopsies from the maximally dilated region of the infrarenal aorta opposite the inferior mesenteric artery as previously described.<sup>14</sup> Tissue collection followed protocols approved by

the ethics committees of the Townsville Hospital and Health Services and James Cook University in line with the Declaration of Helsinki guidelines. For explant culture studies, AAA body biopsies from each patient were dissected into two 100-mg full-thickness explants and stabilized in DMEM+10% fetal bovine serum at 37°C, 5% CO<sub>2</sub>. After 24 hours 1 of the 2 explants was incubated in the presence of the kallikrein-1 neutralizing antibody DX-2300 (50  $\mu$ g/mL; n=6) while the second was incubated in fresh media with an isotype control (n=6), with tissue bradykinin and cyclooxygenase-2, and secreted PGE2 (prostaglandin E2), active MMP2 (metalloproteinase 2), and MMP9 assayed after 72 hours.

## Protein and Activity Analyses

Commercial assays were used as per manufacturer's instruction to measure concentration and activity of target proteins including bradykinin, cyclooxygenase-2, PGE2, total and phosphorylated Akt1 (protein kinase B), total and phosphorylated ERK1/2 (extracellular signal-regulated kinase), MMP2, MMP9, myeloperoxidase, calponin-1, and retinol binding protein-1 in mouse SRA, human AAA tissue, AoSMC, and neutrophil cell culture samples. Assays and corresponding targets are detailed in Table S1. Kallikrein-1 activity was measured by cleavage of the selective substrate D-valyl-L-leucyl-L-arginine *p*-nitroanilide (D-Val-Leu-Arg-pNA; Merck). In a 96-well microplate, 25  $\mu$ L of sample (0.4  $\mu$ g/ $\mu$ L SRA protein from AngII-infused *ApoE*<sup>-/-</sup> mice administered M40-A03 or isotype control) was added to 50  $\mu$ L of assay buffer (0.2 mol/L Tris-HCl, 0.01 mol/L EDTA, pH 8.2) and 25  $\mu$ L of D-Val-Leu-Arg-pNA substrate (0.375 mmol/L). Absorbance at 405 nm was measured at 30-second intervals for 30 minutes (Sunrise Microplate Reader, Tecan). A standard curve was generated using a synthetic form of the reaction product (*p*-nitroaniline, 0–50 nmol; Merck) measured colorimetrically at 420 nm (Sunrise Microplate Reader, Tecan). Kallikrein-1 activity was expressed as micro-units per milligram protein where 1 unit (U) was defined as the amount of kallikrein-1 required to hydrolyze 1  $\mu$ mol of substrate per minute under assay conditions.

## Real-Time Polymerase Chain Reaction

QuantiTect Primer Assays determined gene expression for prostaglandin-endoperoxide synthase 2, also known as cyclooxygenase-2 (*PTGS2*; QT00040586), *CNN1* (QT00067718), *ERK1* (QT00065933), *ERK2* (QT02589321), *AKT1* (QT00085379), Nuclear Factor Kappa B Subunit 1 (*NFKB1*; QT00063791), *MMP2* (QT00088396), and *MMP9* (QT00040040) in human AoSMC using quantitative real time polymerase chain

reaction as previously described.<sup>15</sup> The relative expression of these genes in experimental and control samples was calculated by using the concentration-Ct-standard curve method and normalized using the average expression of GAPDH (human GAPDH, QT00079247) for each sample using the Rotor-Gene Q operating software version 2.0.24. The QuantiTect SYBR Green one-step RT-PCR Kit (Qiagen) was used according to the manufacturer's instructions with 40 ng of total RNA as template. All reactions were independently repeated in duplicate.

### Statistical Analysis

Data were analyzed using GraphPad Prism (version 7) and TIBCO Spotfire S+ (version 8.2). D'Agostino and Pearson's test was used to assess the normality of data and parametric or nonparametric tests applied appropriate to data distribution. Median and interquartile range with maximum and minimum points were presented. Student *t* or Mann-Whitney *U* tests were used for 2-group comparisons of parametric and nonparametric data, respectively. Repeated measures 1-way ANOVA followed by Tukey's multiple comparisons test (parametric) or Kruskal-Wallis test followed by Dunn's multiple comparisons test (nonparametric) were used to compare data within each group obtained as a function of time, with statistical significance between groups (control versus intervention) determined by mixed-effects linear regression. Contingency analyses used Fisher exact test. A *P*<0.05 was considered statistically significant in all cases.

## RESULTS

### Kallikrein-1 Neutralization Limited AngII-Induced SRA Dilatation Associated With Lower SRA Bradykinin Concentration

Baseline mean maximum SRA diameter was similar in control and experimental mice (Figure S1). Both control and experimental mice exhibited a time-dependent increase in SRA diameter in response to AngII infusion (Figure 1A). However, the rate of SRA dilatation was significantly less in mice administered kallikrein-1 neutralizing antibody (Figure 1B). None of the 15 mice receiving kallikrein-1 neutralizing antibody had aortic rupture during the 28-day AngII infusion. In contrast, 5 of the 15 (33%) control mice died of aortic rupture (*P*=0.042; Fisher exact test). Aortas harvested from surviving mice are shown in Figure S2. A significantly lower kallikrein-1 activity and bradykinin concentration were found in the harvested SRA of mice receiving kallikrein-1 neutralizing antibody compared with isotype control (Figure 1C and 1D), which confirmed the efficacy of the neutralizing antibody in the mouse model.

### Kallikrein-1 Neutralization Reduced Cyclooxygenase-2 Activity and Concentrations of PGE2 Within the SRA

Protein samples obtained from the SRA of mice administered either the kallikrein-1 neutralizing antibody or IgG control at completion of the study (day 28) were assayed for cyclooxygenase-2 activity and PGE2 concentration. SRA cyclooxygenase-2 activity was markedly lower in mice administered the kallikrein-1 neutralizing antibody compared with IgG control (Figure 2A). Lower SRA concentrations of PGE2 were also found in mice administered kallikrein-1 neutralizing antibody compared with controls (Figure 2B).

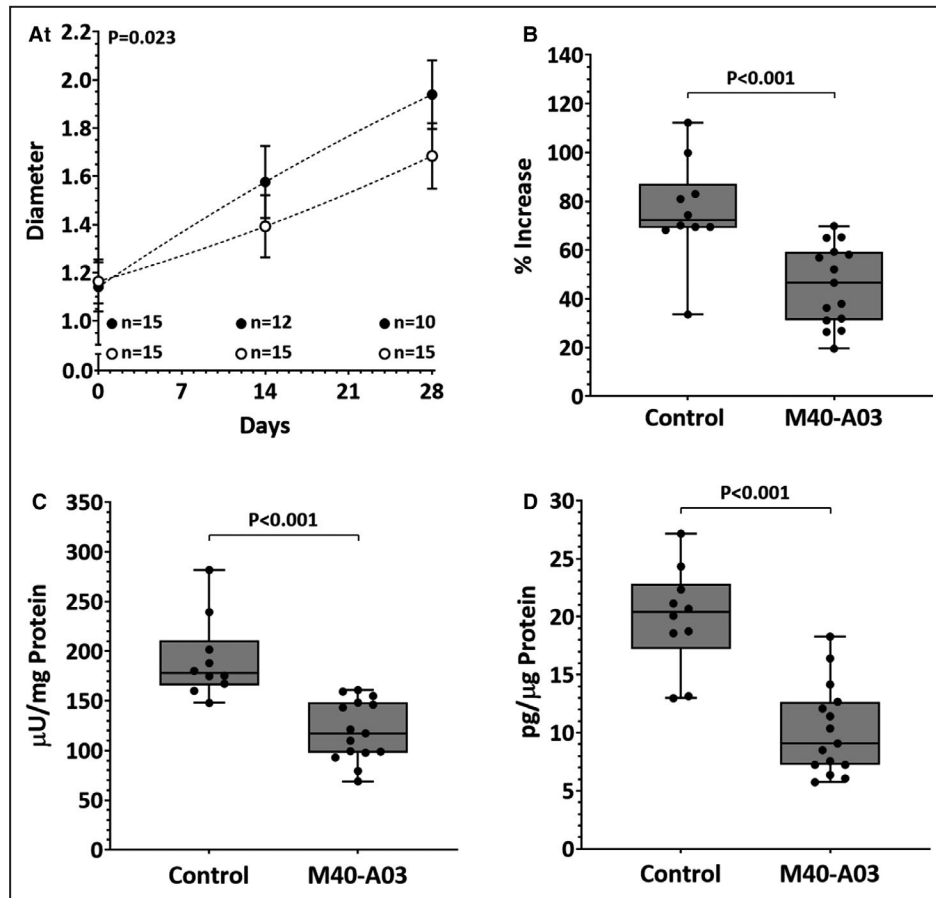
### Akt/Erk Signaling was Downregulated and Active Gelatinases Reduced Within the SRA of Mice Administered Kallikrein-1 Neutralizing Antibody

Protein samples obtained from SRA samples were assayed for Akt1 and Erk1/2 phosphorylation and Mmp2 and Mmp9. The concentrations of total Akt1 and Erk1/2 kinases within the SRA tissue of mice receiving kallikrein-1 neutralizing antibody were similar to concentrations in control mice (Figure S3). In contrast, phosphorylated Akt1 and Erk1/2 were present at significantly lower concentrations (Figure S3). The ratio of phosphorylated-to-total kinase (activity) for both Akt1 and Erk1/2 within SRA tissue from mice administered kallikrein-1 neutralizing antibody was significantly lower than that within SRA of control mice (Figure 2C and 2D). Similarly, SRA concentrations of the active isoform of both Mmp2 and Mmp9 were significantly lower in mice administered kallikrein-1 neutralizing antibody compared with control (Figure 2E and 2F).

### Kallikrein-1 Neutralizing Antibody Reduced Tissue Bradykinin Concentration and Cyclooxygenase-2 Activity and Limited Secretion of PGE2 and Active Gelatinases in Human AAA Explants

Tissue and secreted levels of selected proteins were assessed in human AAA biopsies following 72 hours in explant culture in the presence or absence of human kallikrein-1 neutralizing antibody. Median tissue concentration of bradykinin was 4-fold lower in explants exposed to kallikrein-1 neutralizing antibody compared with control (Table). Cyclooxygenase-2 activity within biopsies incubated with kallikrein-1 neutralizing antibodies was significantly lower compared with IgG controls (Table). Supernatant concentration of PGE2 from biopsies incubated with kallikrein-1 neutralizing antibody was 3-fold lower than control explants (Table). Similarly, secreted levels of active MMP2 and





**Figure 1.** Effect of kallikrein-1 blockade on bradykinin and dilatation of the SRA.

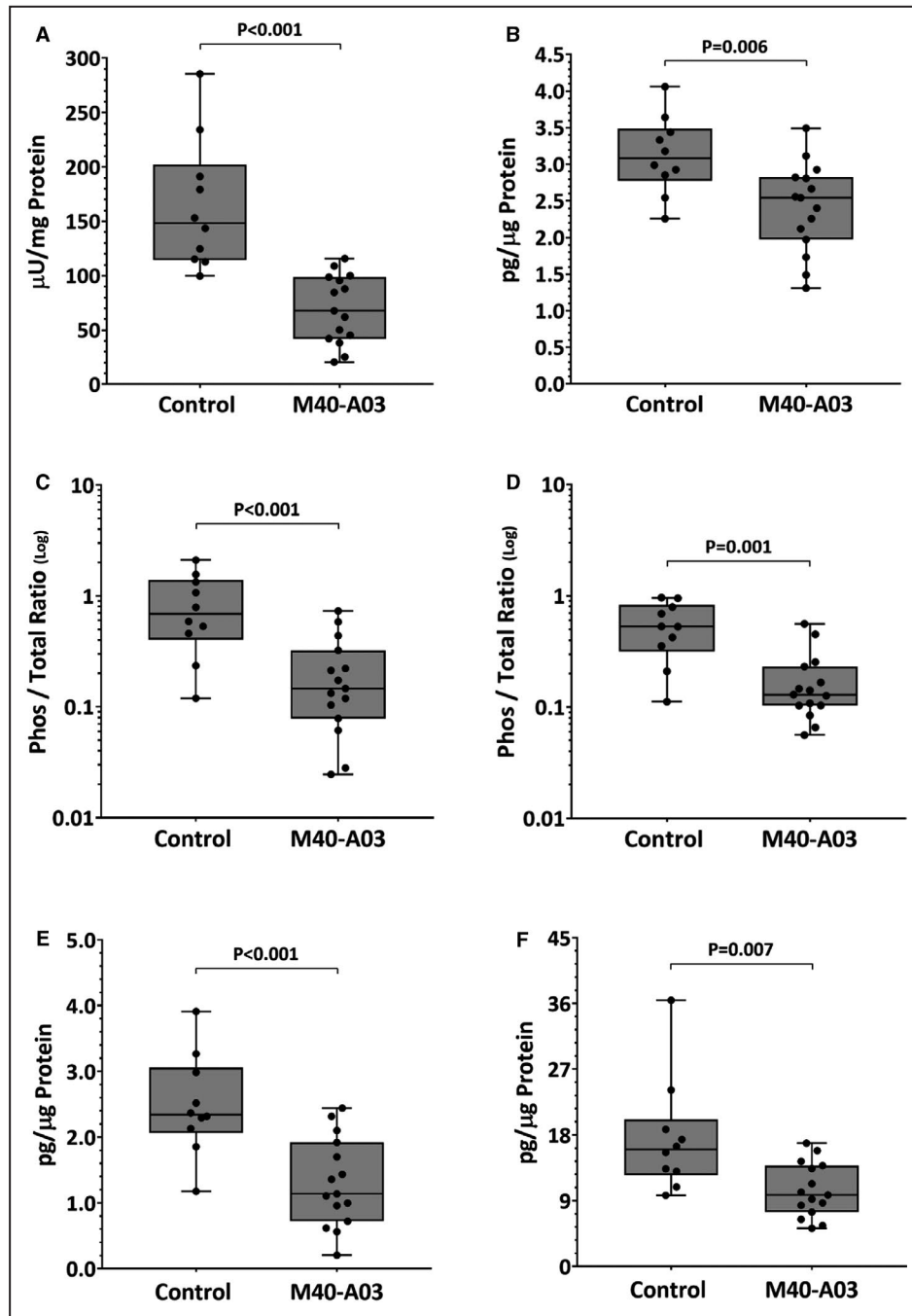
**A**, Ultrasound measurement of the SRA in AngII-infused *ApoE*<sup>-/-</sup> mice administered M40-A03 (open circle) vs isotype control (closed circle). Data expressed as median and interquartile range with maximum and minimum data points (whiskers) for maximum SRA diameter (mm); *P* values calculated for difference between groups by mixed-effects linear regression. **B**, Severity of SRA dilatation at day 28 relative to day 0 (baseline) compared between mice receiving M40-A03 vs isotype control. Data expressed as median and interquartile range with maximum and minimum data points (whiskers) for percent increase in diameter. **C**, Kallikrein-1 activity within the SRA of AngII-infused *ApoE*<sup>-/-</sup> mice receiving the kallikrein-1 neutralizing antibody M40-A03 vs vehicle (control) after 28 days. Data expressed as median and interquartile range with maximum and minimum data points (whiskers) for micro-units ( $\mu$ U) of activity per milligram (mg) SRA protein. **D**, SRA tissue concentration of bradykinin at 28 days in AngII-infused *ApoE*<sup>-/-</sup> mice receiving the kallikrein-1 neutralizing antibody M40-A03 vs vehicle control. Data expressed as median and interquartile range with maximum and minimum data points (whiskers) for picograms (pg) bradykinin per microgram ( $\mu$ g) SRA protein. Two-sided *P* value for comparison between control and experimental by Mann-Whitney *U* test. AngII indicates angiotensin II; *ApoE*<sup>-/-</sup>, apolipoprotein E-deficient mouse; and SRA, suprarenal aorta.

MMP9 were significantly lower in biopsies incubated with kallikrein-1 neutralizing antibody (Table).

### Bradykinin Production in AngII-Stimulated Neutrophils Was Inhibited by Kallikrein-1 Neutralizing Antibody

The action of the human kallikrein-1 neutralizing antibody on AngII-mediated activation of human neutrophils was investigated in vitro. Culture supernatants were assayed for myeloperoxidase, a hallmark indicator

of the neutrophil respiratory burst, as a measure of neutrophil activation. Myeloperoxidase concentration within supernatants of neutrophils exposed to AngII was 5-fold above resting cells ( $P=0.002$ ; Figure S4). Supernatant myeloperoxidase in AngII-stimulated neutrophils co-incubated with kallikrein-1 neutralizing antibody remained higher than resting cells and comparable to neutrophils stimulated with AngII alone (Figure S4). Bradykinin production in AngII-stimulated neutrophils, however, was decreased 2-fold upon co-incubation with kallikrein-1 neutralizing antibody (Table S2).



**Figure 2.** Effect of kallikrein-1 neutralization on cyclooxygenase-2, PGE2, Akt, Erk, and Mmp activity.

Cyclooxygenase-2 activity (A) and concentration of PGE2 (B) within the SRA of AngII-infused *ApoE*<sup>-/-</sup> mice administered M40-A03 vs vehicle (control) after 28 days. Data expressed as median and interquartile range with maximum and minimum data points (whiskers) for micro-units (μU) of activity per milligram (mg) SRA protein, or picograms (pg) per microgram (μg) SRA protein, respectively. Phosphorylation of Akt1 (C) and Erk1/2 (D) in SRA of AngII-infused *ApoE*<sup>-/-</sup> mice administered M40-A03 vs vehicle (control) after 28 days. Data expressed as median and interquartile range with maximum and minimum data points (whiskers) for quantitative relationship (ratio) between phosphorylated and total kinase. Concentration of active Mmp2 (E) and active Mmp9 (F) within the SRA of *ApoE*<sup>-/-</sup> mice administered M40-A03 vs vehicle (control) after 28 days. Data expressed as median and interquartile range with maximum and minimum data points (whiskers) for nanograms (ng) of active Mmp per microgram (μg) SRA protein. Two-sided *P* value for comparison between control and experimental by Mann-Whitney *U* test. AngII indicates angiotensin II; Akt, protein kinase B; *ApoE*<sup>-/-</sup>, apolipoprotein E-deficient mouse; Erk, extracellular signal-regulated kinase; Mmp, matrix metalloproteinase; PGE2, prostaglandin E2; and SRA, suprarenal aorta.

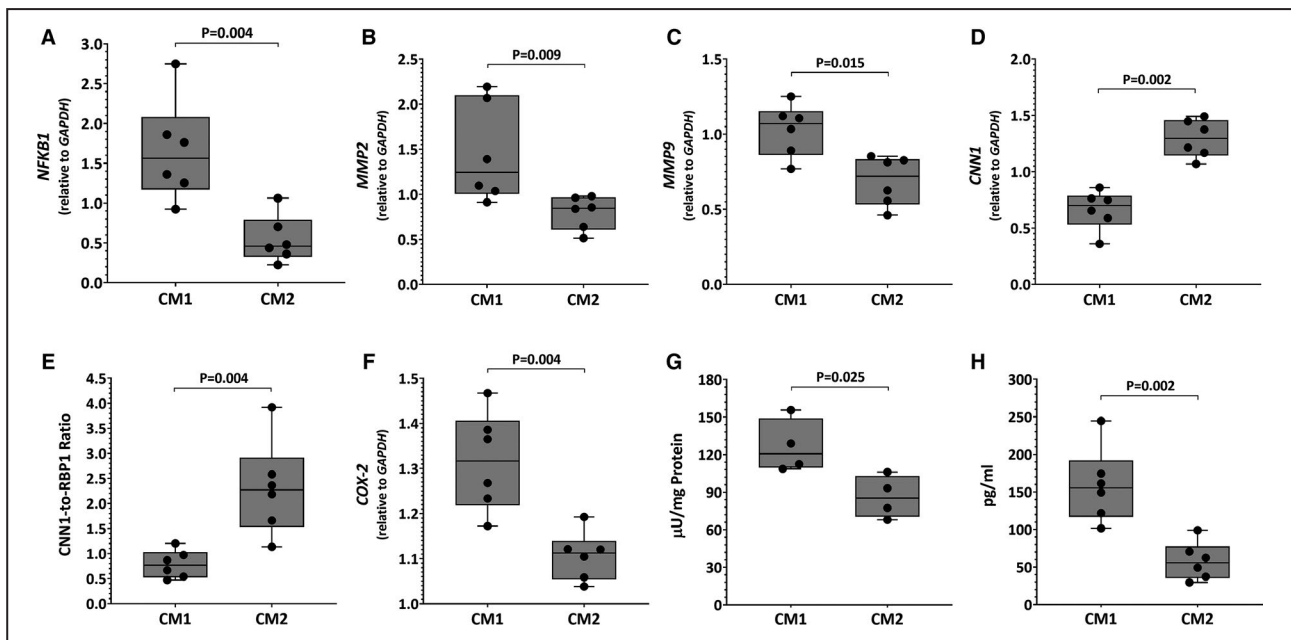
## Response of Human AoSMC to Conditioned Media From AngII-Stimulated Neutrophils Incubated in the Presence or Absence of Kallikrein-1 Neutralizing Antibody

Human AoSMC were incubated over 24 hours in control media, control media supplemented with 10% vol/vol conditioned media from AngII-activated neutrophils (CM1), or control media supplemented with AngII-activated neutrophils co-incubated with kallikrein-1 neutralizing antibody (CM2). Gene and protein expression associated with AoSMC phenotype and function was assessed. Exposure of AoSMC to CM1 stimulated an inflammatory response marked by a 10-fold upregulation in gene expression for nuclear factor kappa-light-chain-enhancer of activated B cells and downregulation of the differentiation marker calponin-1 compared with control cells (Figure S5). Incubation of AoSMC with CM2 over the same period resulted in a 3-fold decrease in nuclear factor kappa-light-chain-enhancer of activated

B cells and concomitant downregulation in MMP2 and MMP9 expression compared with CM1-exposed cells (Figure 3A through 3C). Gene expression for calponin-1 was upregulated 2-fold and corresponded with a 3-fold increase in calponin-1-to-retinol binding protein-1 protein ratio in AoSMC incubated with CM2 compared with CM1 (Figure 3D and 3E). In contrast, gene expression for cyclooxygenase-2 was markedly downregulated in AoSMC incubated with CM2 and associated with significant decrease in cyclooxygenase-2 activity and a 3-fold reduction in PGE2 concentration in these cells (Figure 3F through 3H).

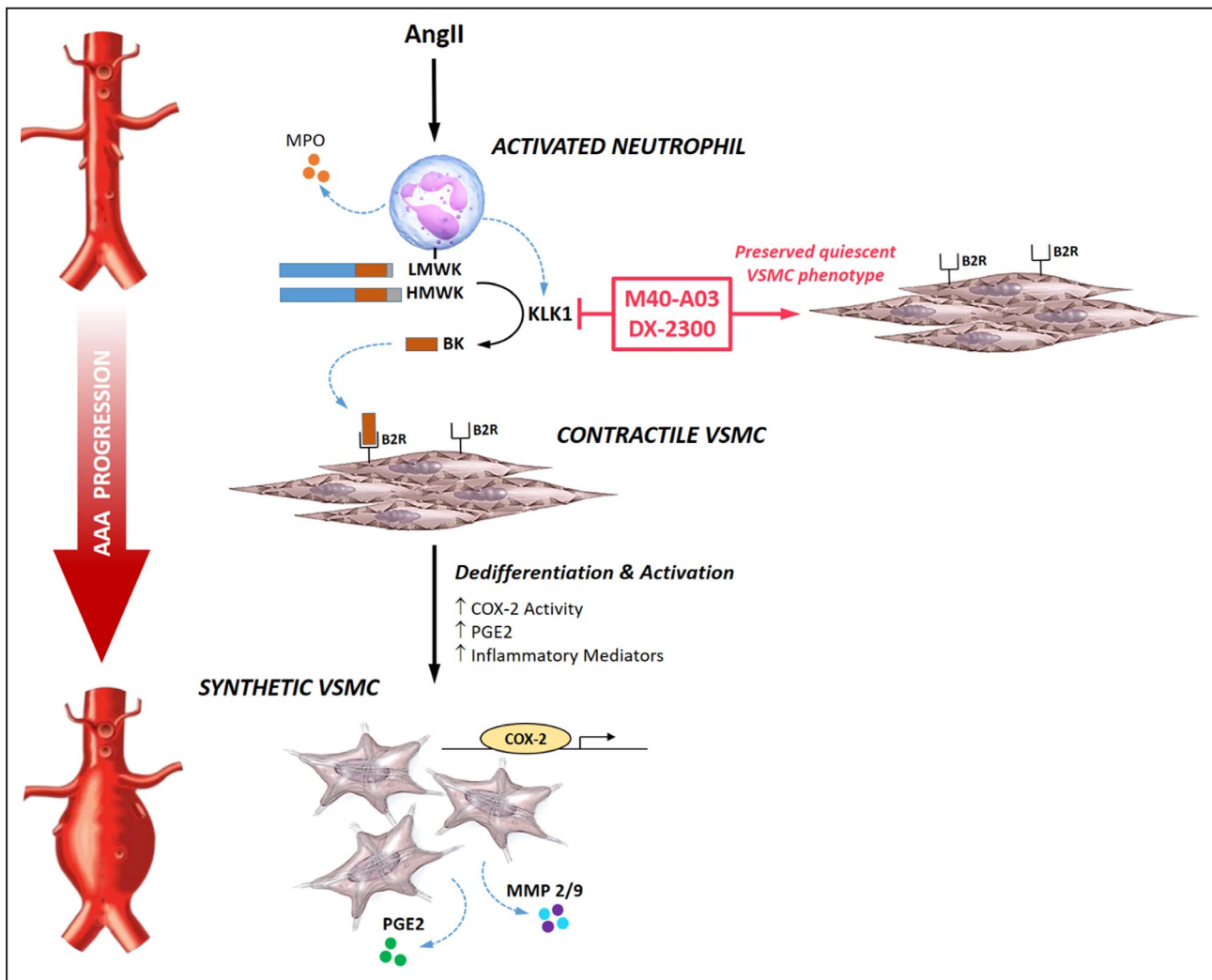
## DISCUSSION

The main finding of this study was that kallikrein-1 neutralization blocked several processes strongly implicated in AAA pathogenesis, including cyclooxygenase-2 and MMP activity. These effects were consistent in a mouse model, human AAA explants, and AoSMC. The effects of kallikrein-1 neutralization translated into significantly limiting the size of the AAAs



**Figure 3.** In vitro response of human AoSMCs to conditioned media from AngII-activated human neutrophils.

Human AoSMC were incubated over 24 hours in media supplemented with 10% vol/vol conditioned media from AngII-activated neutrophils (CM1) or media supplemented with AngII-activated neutrophils co-incubated with kallikrein-1 neutralizing antibody (CM2). **A** through **C**, Gene expression for pro-inflammatory markers NFKB1, MMP2, and MMP9. Data expressed as median and interquartile range with maximum and minimum data points (whiskers) for gene expression relative to *GAPDH*. **D**, Gene expression for contractile (quiescent) smooth muscle cell phenotype marker CNN1. Data expressed as median and interquartile range with maximum and minimum data points (whiskers) for gene expression relative to *GAPDH*. **E**, Protein ratio for contractile phenotype marker CNN1 to synthetic phenotype marker RBP1 as a measure of phenotype status. Data expressed as median and interquartile range with maximum and minimum data points (whiskers) for picograms of CNN1-to-RBP1 (ratio) per microgram of protein. **F** through **H**, Gene expression (**F**) and cellular activity for cyclooxygenase-2 (**G**) and secreted levels of PGE2 (**H**). Data expressed as median and interquartile range with maximum and minimum data points (whiskers) for gene expression relative to *GAPDH*, micro-units ( $\mu$ U) of activity per milligram (mg) of protein and picograms (pg) per milliliter (mL) supernatant. Two-sided *P* value for comparison between control and experimental groups by Mann-Whitney *U* test. AngII indicates angiotensin II; AoSMC, aortic smooth muscle cell; CNN1, calponin-1; MMP, matrix metalloproteinase; NFKB1, nuclear factor kappa-light-chain-enhancer of activated B cells; PGE2, prostaglandin E2; and RBP1, retinol binding protein-1.



**Figure 4. Potential pathways by which inhibition of kallikrein-1 may limiting AAA pathogenesis.**

Schematic illustrating the potential relationship between vascular smooth muscle cell (VSMC) phenotypic switching induced by neutrophil-derived bradykinin (BK) stimulation of the kinin B2 receptor (B2R) and AAA. BK-mediated upregulation of cyclooxygenase-2 gene expression and activity in dedifferentiated VSMC is associated with increased PGE2 and production of active MMP2 and MMP9. Blockade of the neutrophil kallikrein-kinin pathway and subsequent BK generation via kallikrein-1-neutralizing antibodies (M40-A03, DX-2300) limits VSMC dedifferentiation, preserving the quiescent (contractile) phenotype. AAA indicates abdominal aortic aneurysm; AngII, angiotensin II; COX-2, cyclooxygenase-2; HMWK, high molecular weight Kininogen; KLK1, kallikrein-1; LMWK, low molecular weight kininogen; MMP, matrix metalloproteinase; MPO, myeloperoxidase; and PGE2, prostaglandin E2.

that developed and the number of AAAs that ruptured in a mouse model. Overall, the findings suggest that blocking kallikrein-1 is a potential target to limit AAA progression.

Genome-wide DNA linkage analyses of families in which 2 or more members had an AAA previously identified a genomic region on chromosome 19q13 to be associated with AAA.<sup>16,17</sup> This region contains the gene encoding kallikrein-1.<sup>16</sup> In a subsequent study, a single nucleotide mutation in exon 3 of the kallikrein-1 gene was reported to be associated with large AAA.<sup>18</sup> In a prior experimental study, it was found that an agonist for the B2 kinin receptor promoted the ability of AngII to induce AAA development and rupture.<sup>8</sup> Conversely, a B2 kinin antagonist inhibited AngII or

calcium phosphate-induced aortic expansion.<sup>8</sup> These findings in a mouse model and patients identified kinins as potentially involved in AAA pathogenesis.

Kinins can be generated from kininogen by both plasma and tissue kallikrein. Plasma kallikrein is released from pre-kallikrein by activated Factor XII. It was previously reported that Factor XII deficiency or inhibition significantly reduced AngII-induced aortic expansion in *ApoE*<sup>-/-</sup> mice.<sup>12</sup> The current study adds to this prior research by showing that the inhibition of kallikrein-1 also significantly limited AAA development. Since kallikrein-1 is expressed directly in the aorta and potentially has less wide-ranging effects than plasma kallikrein, it seems likely that it may be a safer target for a potential AAA drug.



**Table. Effect of Kallikrein-1 Neutralization With DX-2300 on Bradykinin and Matrix Remodeling Proteins in Human AAA Biopsies**

	N	Control	DX-2300	P Value
Bradykinin (tissue) <sup>†</sup>	6	1.61 (0.85–5.26)	0.40 (0.31–0.74)	0.009
Cyclooxygenase-2 (tissue) <sup>†</sup>	4	153.1 (140.7–245.2)	101.8 (92.7–107.5)	0.029
PGE <sub>2</sub> (secreted) <sup>‡</sup>	6	1.96 (1.15–2.60)	0.60 (0.28–0.96)	0.015
actMMP2 (secreted) <sup>§</sup>	6	2.46 (1.85–2.82)	1.44 (1.01–1.76)	0.026
actMMP9 (secreted) <sup>§</sup>	6	2.25 (2.03–2.66)	1.33 (1.00–1.62)	0.002

P, 2-sided P value for comparison between control and experimental by Mann–Whitney U test. AAA indicates abdominal aortic aneurysm; actMMP, active form of metalloproteinase; and PGE<sub>2</sub>, prostaglandin E<sub>2</sub>.

Data expressed as median (interquartile range) nanograms per microgram protein,<sup>†</sup> micro-units of activity per milligram protein,<sup>‡</sup> picograms per milliliter supernatant,<sup>§</sup> or relative density units per microgram protein.<sup>§</sup>

Past mouse model and human research has implicated cyclooxygenase-2 in AAA pathogenesis.<sup>19–25</sup> Both cyclooxygenase-2 deficiency<sup>22</sup> and inhibition<sup>20,23</sup> have been reported to inhibit AngII-induced AAA formation. Nonselective cyclooxygenase inhibition has also been reported to inhibit elastase perfusion-induced AAA formation.<sup>24</sup> The expression of cyclooxygenase-2 and secretion of PGE<sub>2</sub> have been reported to be greater in samples from ruptured compared with intact human AAAs.<sup>19,25</sup> In these previous studies, cyclooxygenase-2 has been identified both in infiltrating inflammatory cells and AoSMC.<sup>21,22</sup> Bradykinin has been previously reported to upregulate cyclooxygenase-2 expression and activity within AoSMC and neutrophil cell lines in vitro.<sup>26,27</sup> This past research suggested that kinins might promote AAA via upregulating cyclooxygenase-2 and secretion of PGE<sub>2</sub>.

In the current study, kallikrein-1 neutralization was effective in decreasing bradykinin levels in both the AngII mouse model and human AAA explants. This was associated with reduced cyclooxygenase-2 expression and activity, and reduced production of PGE<sub>2</sub> and active MMP2 and MMP9. These effects were also found when neutrophils were exposed to kallikrein-1 neutralization and subsequently incubated with AoSMC, suggesting that these cells were an important source of bradykinin, as previously described.<sup>28</sup> Neutrophils possess the complete apparatus for synthesis and release of kinins.<sup>29</sup> Kallikrein-1 released by activated neutrophils converts kininogen on the neutrophil surface to bradykinin, promoting inflammation.<sup>29</sup> Decreased production of bradykinin within the SRA, because of neutralization of neutrophil kallikrein-1, likely limited B2 receptor-mediated AoSMC activation and AngII-stimulated aortic remodeling. Support for this theory was provided by the results of the in vitro study, which showed that bradykinin production by AngII-stimulated neutrophils was blocked in the presence of the kallikrein-1 neutralizing antibody. Furthermore, phenotype switching of AoSMC, evidenced by upregulation of *CNN1* and increased calponin-1:retinol binding protein-1 ratio and downregulation of NFκB1, MMP2, and MMP9, was inhibited by kallikrein-1 neutralizing antibody. Overall, the

findings of the current study and prior investigations suggest that blocking neutrophil-derived kinins is a potential target to limit AoSMC phenotypic switching and aortic inflammation and remodeling believed to be key in AAA pathogenesis as outlined in Figure 4.<sup>8</sup>

The current study has several strengths and limitations. Strengths include the combination of mouse model, human sample explant, and in vitro research. Limitations include that only 1 mouse model was studied. A control group that did not receive angiotensin II was not included in the current study since the focus was on testing kallikrein-1 blockade during advanced aortic pathology. Tissue samples examined in the current study were collected from the end of the experiment. It is therefore possible that effects of the kallikrein-1 inhibition at an earlier stage of aneurysm development might have been missed. Furthermore, gene expression and protein quantification were used to examine aortic samples. Samples were not examined by histology, which might have provided additional experimental insight. Also, it remains unproven beyond doubt that the benefits of kallikrein-1 neutralization identified were directly caused by cyclooxygenase-2 inhibition. Other mechanisms not studied in this investigation might also have contributed. It should also be noted that the reported findings remain experimental and their translation to patients remains to be proven.

In conclusion, the current study, building on past evidence, suggests that kallikrein-1 neutralization through reducing generation of bradykinin and actions of cyclooxygenase-2 limit AAA development in a mouse model induced through AngII infusion.

## ARTICLE INFORMATION

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Author contributions: Moran designed and performed the mouse work, analyzed the data, and drafted the manuscript. Biros performed molecular analyses and data analyses. Krishna and Morton assisted with the animal model and performed protein analyses. Sexton provided the mouse (M40-A03) and human (DX-2300) kallikrein-1 inhibitors and corresponding IgG controls used in the study. Golledge co-designed the studies, obtained funding, and contributed to data analysis and interpretation, and co-drafted the manuscript and edited it. All authors contributed to critical revision of the manuscript.

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## Disclosures

Dan Sexton was an employee of Shire Pharmaceuticals (USA) and now works for Takeda. The remaining authors have no disclosures to report.

## Supplementary Material

Tables S1–S2

Figures S1–S5

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# SUPPLEMENTAL MATERIAL

**Table S1. Commercial assays used in this study.**

<b>Model/Source</b>	<b>Target</b>	<b>Assay</b>	<b>Manufacturer</b>
Mouse SRA	Bradykinin	ELISA; ADI-900-206	Enzo Life Sciences
	COX-2 Activity	Enzyme; 760151	Cayman Chemical
	PGE <sub>2</sub>	ELISA; ab133021	abcam
	Akt1	ELISA; ab176657	abcam
	Erk1/2	ELISA; ab176660	abcam
	Active MMP2	Enzyme; QZBmmp2M	QuickZyme Biosciences
	Active MMP9	Enzyme; QZBmmp9M	QuickZyme Biosciences
Neutrophil <sup>†</sup>			
<i>Secreted</i>	MPO Activity	EnzChek <sup>®</sup> ; E33856	ThermoFisher Scientific
	Bradykinin	ELISA; ADI-900-206	Enzo Life Sciences
AoSMC <sup>†</sup>			
<i>Lysate</i>	CNN1	ELISA; OKCD09197	Aviva Systems Biology
	RBP1	ELISA; OKCD06311	Aviva Systems Biology
	COX-2 Activity	Enzyme; 760151	Cayman Chemical
<i>Secreted</i>	PGE <sub>2</sub>	ELISA; ab133021	abcam
Human AAA <sup>‡</sup>			
<i>Homogenate</i>	Bradykinin	ELISA; ADI-900-206	Enzo Life Sciences
	COX-2 Activity	Enzyme; 760151	Cayman Chemical
<i>Secreted</i>	PGE <sub>2</sub>	ELISA; ab133021	abcam

*SRA, suprarenal aorta; PGE<sub>2</sub>, prostaglandin E<sub>2</sub>; Akt1, total and phosphorylated protein kinase B; Erk1/2, total and phosphorylated extracellular signal-regulated kinase 1/2; MMP, metalloproteinase; MPO, myeloperoxidase; AoSMC, aortic smooth muscle cell; CNN1, calponin-1; RBP1, retinol binding protein 1; COX-2, cyclooxygenase-2; <sup>†</sup>human cell; <sup>‡</sup>biopsy explant.*

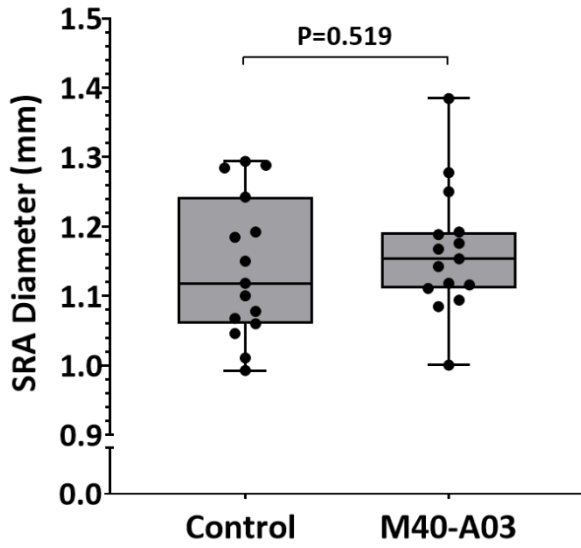


**Table S2. Effect of kallikrein-1 neutralisation (DX-2300) on AngII-stimulated activation and bradykinin production in human neutrophils *in vitro*.**

Supernatant	AngII	AngII + DX-2300		P
n (cultures)	6	6		
MPO <sup>†</sup>	0.43 (0.39-0.51)	0.37 (0.34-0.43)	-	0.076
Bradykinin <sup>‡</sup>	108 (100-114)	76 (68-92)	↓	0.004

*AngII, angiotensin II; MPO, myeloperoxidase; Data expressed as median (interquartile range) relative fluorescence units per microgram protein<sup>†</sup>, or picograms per microgram protein<sup>‡</sup>; P, two-sided p-value for comparison between control and experimental by Mann-Whitney U test.*

Figure S1. Baseline (prior to AngII infusion) suprarenal aortic diameter at peak systole measured by ultrasound in *ApoE*<sup>-/-</sup> mice allocated to control and intervention (M40-A03) groups.



**Figure S2. Gross morphology of aortas harvested from AngII-infused ApoE<sup>-/-</sup> mice administered kallikrein-1-neutralising antibody (M40-A03) or isotype (control) over 28 days.**

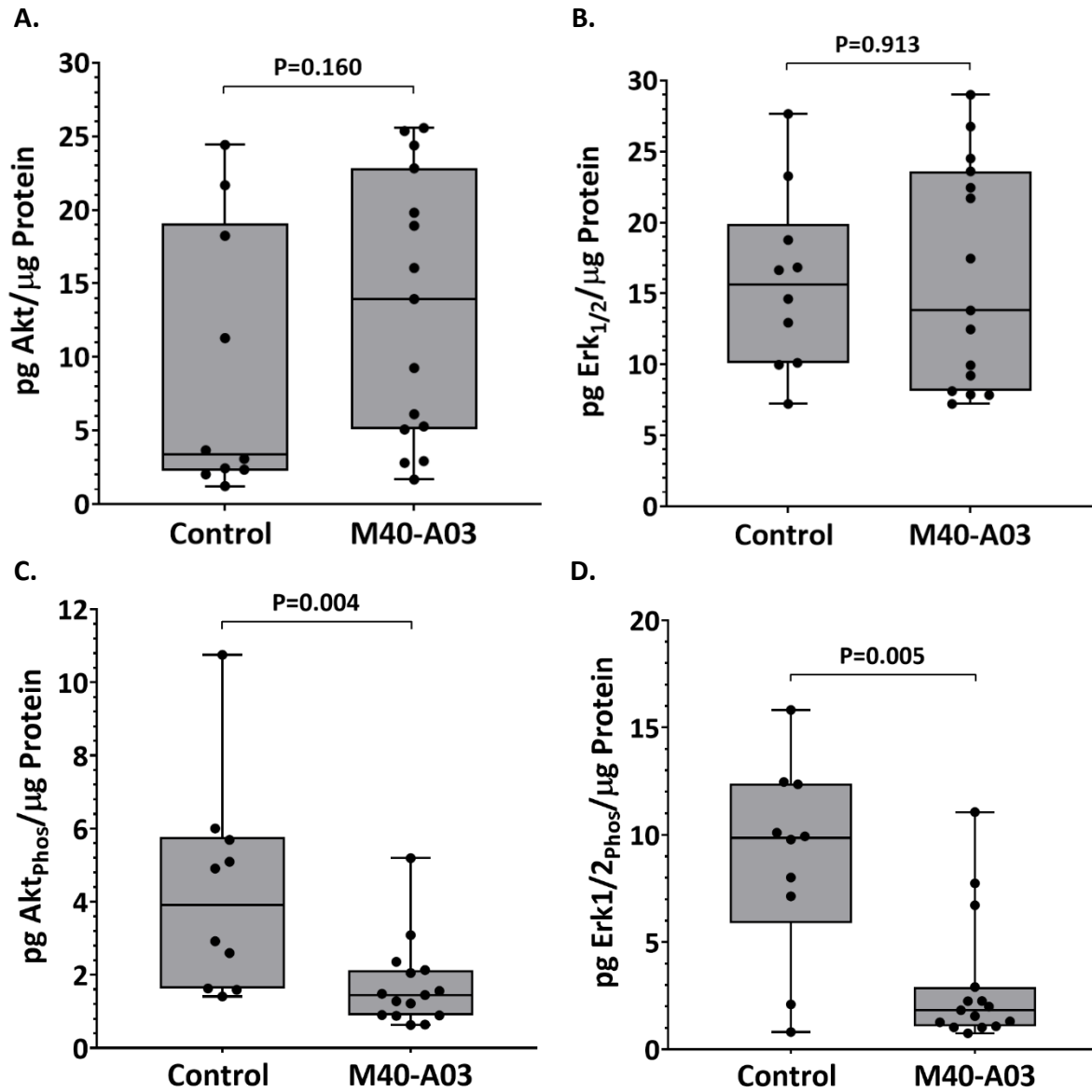
**Control**



**M40-A03**

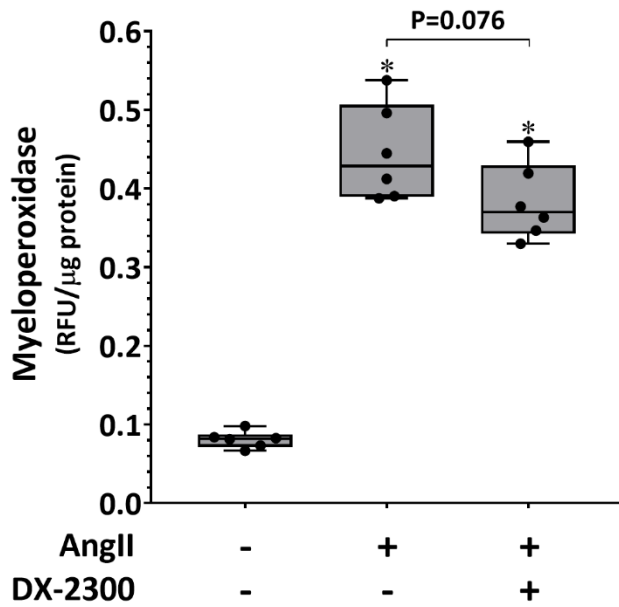


**Figure S3. Protein concentration of total protein kinase B (Akt; A), total extracellular signal-regulated kinase (Erk)1/2 (B), phosphorylated Akt (C), and phosphorylated Erk 1/2 (D) kinases within SRA tissue of mice receiving kallikrein-1-neutralising antibody (M40-A03) or isotype (control) after 28 days.**



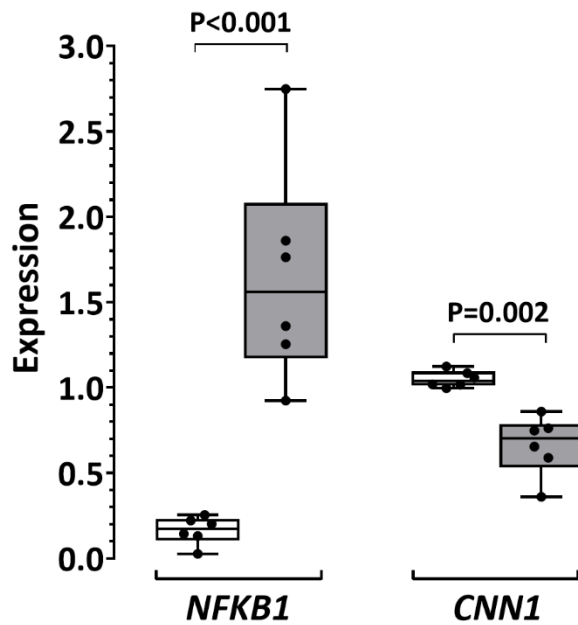


**Figure S4. Effect of kallikrein-1 neutralisation on neutrophil activation.**



Myeloperoxidase (MPO) activity assessed in supernatant of human neutrophils cultured for 30 minutes in the presence/absence of AngII (10 nM) or the kallikrein-1 neutralising antibody DX-2300 (5  $\mu\text{g/ml}$ ). Data expressed as median and interquartile range with maximum and minimum data points (whiskers) for relative fluorescence units (RFU) per  $\mu\text{g}$  protein from six repeat cultures. \* $P < 0.05$  compared to control by ANOVA with Tukey's post-hoc test for multiple comparisons.

**Figure S5. Stimulation of an inflammatory phenotype in human aortic smooth muscle cells (AoSMC).**



mRNA expression for pro-inflammatory marker nuclear factor kappa-light-chain-enhancer of activated B cells (*NFKB1*) and quiescent phenotype marker calponin-1 (*CNN1*) in AoSMC following incubation over 24 hours in 10% v/v conditioned media derived from AngII-stimulated neutrophil cultures (grey bar), compared to control media (white bar). Data expressed as median and interquartile range with maximum and minimum data points (whiskers) for gene expression relative to *GAPDH*.