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Abstract

Coarctation of the aorta (CoA) is a common congenital cardiovascular (CV) defect characterized by a stenosis of the descending thoracic aorta. Treatment exists, but many patients develop hypertension (HTN). Identifying the cause of HTN is challenging because of patient variability (e.g., age, follow-up duration, severity) and concurrent CV abnormalities. Our objective was to conduct RNA sequencing of aortic tissue from humans with CoA to identify a candidate gene for mechanistic studies of arterial dysfunction in a rabbit model of CoA devoid of the variability seen with humans. We present the first known evidence of natriuretic peptide receptor C (*NPR-C*; aka *NPR3*) downregulation in human aortic sections subjected to high blood pressure (BP) from CoA versus normal BP regions (validated to PCR). These changes in *NPR-C*, a gene associated with BP and proliferation, were replicated in the rabbit model of CoA. Artery segments from this model were used with human aortic endothelial cells to reveal the functional relevance of altered NPR-C activity. Results showed decreased intracellular calcium ($[Ca^{2+}]_i$) activity to C-type natriuretic peptide (CNP). Normal relaxation induced by CNP and atrial natriuretic peptide was impaired for aortic segments exposed to elevated BP from CoA. Inhibition of NPR-C (M372049) also impaired aortic relaxation and $[Ca^{2+}]_i$ activity. Genotyping of *NPR-C*variants predicted to be damaging revealed that rs146301345 was enriched in our CoA patients, but sample size limited association with HTN. These results may ultimately be used to tailor treatment for CoA based on mechanical stimuli, genotyping, and/or changes in arterial function.

INTRODUCTION

Excessive mechanical forces such as blood pressure (BP), wall tension, and strain contribute to pathologic remodeling of the aorta and arterial system (22, 39), which can lead to substantial morbidity (24). Morbidity in the form of hypertension (HTN) is common in children with coarctation of the aorta (CoA), one of the most common congenital cardiovascular (CV) defects that is characterized by a constriction of the descending thoracic aorta. Currently there is treatment, but no cure for CoA. The cause of HTN in CoA patients remains unknown despite the first surgical treatment being conducted ~75 yr ago (18). Genetic factors are believed to be associated with CoA and may contribute to persistent morbidity after treatment (14, 33). However, the specific genes contributing to the pathology of CoA, and their functional relevance, remain unknown.

Identifying the mechanisms of morbidity is difficult in humans with CoA due to confounding variables such as differences in age at repair, time between correction and follow-up, severity of CoA before correction, and concurrent anomalies (e.g., bicuspid aortic valves or septal defects). While there is likely a causal genetic basis for the initial presentation of CoA in humans, there are also likely changes in gene expression resulting from mechanical stimuli on the vasculature as a result of the CoA shortly after birth (9, 16). To address this complexity, we previously developed a rabbit model that allows us to study the mechanical consequences of CoA independently of the confounding variables mentioned above (26). This model replicates aortic changes in humans and mimics correction at various durations using dissolvable suture (Fig. 1). Our results to date using a putative clinical treatment guideline (20 mmHg BP gradient) with this model have revealed increased medial wall thickness and stiffening, a phenotypic shift in smooth muscle cells (SMC) to the dedifferentiated state, and endothelial dysfunction as evident by decreased nitric oxide (NO) relaxation (via acetylcholine stimulation), all of which persist after correction (25). Microarray analysis of aortic tissue from this model identified several differentially expressed genes (DEG) in the region exposed to pronounced mechanical stimuli developing from CoA (19). The objective of the current investigation was to use RNA sequencing (RNA-Seq) of aortic tissue from humans with CoA to identify one or more candidate genes for use in future mechanistic studies of coarctation induced arterial dysfunction. We then sought to determine the association of any candidate genes with the likelihood of HTN in a larger cohort of patients with CoA from our center. Specifically, we present the first known evidence of natriuretic peptide receptor C (*NPR-C*; also known as *NPR3*) downregulation in humans treated for CoA and use our rabbit model of CoA to show the functional relevance of coarctation induced changes in *NPR-C*.

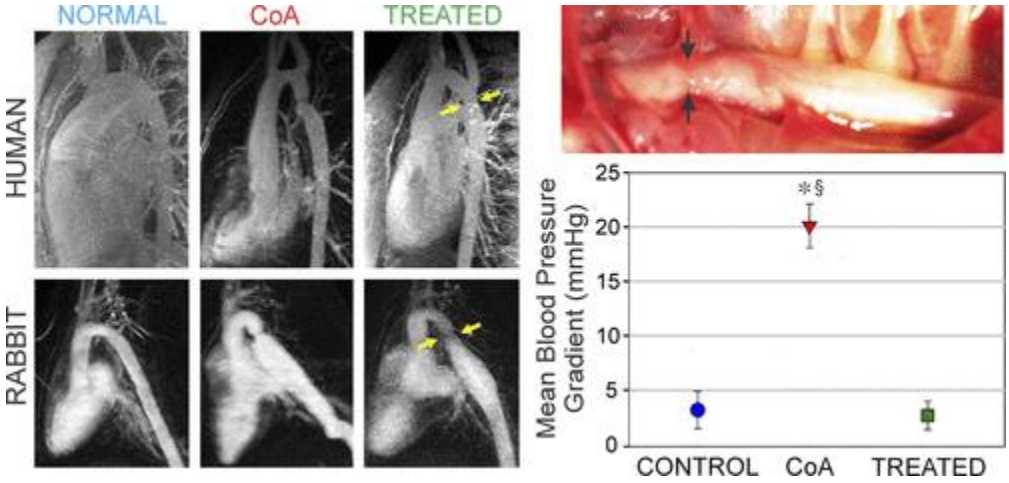

Fig. 1.Magnetic resonance images showing the similarity between untreated and treated coarctation of the aorta (CoA) in humans (left top) vs. a rabbit model (left bottom) developed to study mechanisms of arterial dysfunction in the absence of concurrent anomalies. Yellow arrows denote treatment sites. The right images show CoA suture (black arrows) and resulting blood pressure gradient (BPG) for groups of juvenile rabbits (n =

7/group) with 20 mmHg BPG. Symbols indicate significantly ($P < 0.05$) different from Control (*) and Treated (§) groups.

METHODS

All procedures were reviewed and approved by the Institutional Review Board and Institutional Animal Care and Use Committee of the Children's Hospital of Wisconsin, Medical College of Wisconsin, and Marquette University as previously described (26, 34).

Human RNA sequencing and genotyping.

Samples for the current study were extracted from the Congenital Heart Disease Tissue Bank at our institution for two purposes. Sections from above (proximal; high BP region) and below (distal; low BP region) the coarctation were extracted from patients for RNA-Seq and transcriptome analysis. White blood cell DNA from 242 CoA patients was subsequently used for genotyping as described in more detail below. All tissue was obtained after consent and at the time of corrective surgery.

Transcriptome isolation.

RNA-Seq analysis of samples was performed similar to details described previously (35). Briefly, aortic tissue for RNA-Seq was selected from six CoA patients (average age = 6.5 yr, range 0.31–11 yr; Table 1) with an upper extremity systolic BP >99th percentile for their sex, height, and age according to the National High Blood Pressure Education Program (NHBPEP) Working Group on Children and Adolescents (27a), thereby focusing on mechanical consequences imposed by the coarctation. RNA was isolated from aortic samples (~10 mg) by enzymatic digestion with an Ambion MELT kit (Invitrogen), which includes an "on-bead" DNase digestion step to remove contaminating genomic DNA. Samples were homogenized with glass tissue grinders needed for fibrous samples. The Qiagen RNeasy Fibrous Minikit (Qiagen; Valencia, CA) was then used according to the manufacturer's instructions. Isolated RNA was of high quality as determined by Bioanalyzer 2100 RNA integrity number (Table 1; Agilent Technologies). RNA sequencing libraries were prepared from 500 ng total RNA using the Illumina TruSeq kit (version 2.5). Samples were spiked with external RNA controls (ERCC sequences; Ambion Life Technologies, Grand Island, NY). Unique indexes were introduced according to the protocol in order for sample multiplexing during the sequencing run. Library quantitation was accomplished by quantitative (q)PCR, and subsequent sequencing was carried out on an Illumina HiSeq 2000 platform. Approximately 35 million paired-end reads were generated per sample.

Table 1. Patient characteristics, sample locations, and RNA quality

Subject	Age, yr	Sex	Preop BP, systolic/diastolic	Sample Location	RNA Integrity #	Matched Pair (yes = X)
1	5.6	M	139/91	proximal	8.3	X
				distal	8.2	
2	9.3	M	126/71	proximal	7.6	X
				distal	8.7	
3	0.3	M	118/69	proximal	7.0	X
				distal	8.2	
4	8.0	F	133/58	proximal	8.0	X
				distal	8.1	
5	11	F	134/80	proximal	7.4	
6	5.2	M	130/89	distal	7.8	
Average (SD)	6.5 (3.7)			Average (SD)	7.9 (0.49)	

BP, blood pressure.

Transcriptome data analysis.

Illumina HiSeq 2000 paired-end reads were mapped to the human genome (NCBI b37) using gapped alignment software Bowtie (36) under RSEM 1.2.7. Quantification to posterior mean estimate transcripts per million (TPM) was performed by RSEM (http://deweylab.biostat.wisc.edu/rsem/) across a transcriptome reference of 38,642 from RefSeq (genes, alternative transcripts, mitochondrial sequences, and spiked-in ERCC controls). Transcripts were analyzed with EdgeR by scaling TPM to produce pseudocounts, calculating normalization factors, then estimating GLM common, trended, and tagwise dispersions. EdgeR is a standard package of the R programming language for differential expression analysis. A likelihood ratio test evaluated each transcript's model significance by tissue location (proximal vs. distal). The generalized linear modeling functionality in EdgeR was used to perform paired samples test (31). A *P* value < 0.05 was considered statistically significant, yielding DEG. Results were filtered to retain those transcripts with absolute log fold change and expression (logCPM) greater than one. EdgeR results were processed by removing DEG within the proximal vs. distal sections for any individual subject, thereby leaving only those DEG with significant changes in expression across all patients.

DEG from the RNA-Seq analysis of aortic tissue from humans with CoA above were compared with those from a prior study generating DEG from proximal aortic tissue of untreated CoA, treated CoA, or control rabbits (19). Three common DEG emerged: DENN domain containing 2D (*DENND2D*), ArfGAP with coiled-coil, ankyrin repeat and PH domains 1 (*ACAP1*), and *NPR-C*. In contrast to *DENND2D* and *ACAP1*, a comprehensive literature search suggested a potential role for *NPR-C* in the vasculature (3, 5, 15, 21). *NPR-C* was therefore determined to be the most promising and pragmatic candidate for more detailed functional analysis in the rabbit model of CoA and cell culture approaches. The relative quantification of *NPR-C* vs. GAPDH was then compared between RNA-Seq results and qPCR (TaqMan Assay ID: Hs01099013_m1).

Genotyping for NPR-C variants.

At the time of study, there were five known single-nucleotide polymorphisms (SNPs) for *NPR-C* that are associated with high systolic BP or HTN (Table 2). We identified one additional *NPR-C* variant (Table 2; rs146301345) from a population of ~200 patients whose tissue underwent whole exome or whole genome sequencing at our center previously (13, 35). These patients had hypoplastic left heart syndrome (HLHS), which is a congenital cardiovascular disease often including CoA (67–80%) (12).

Table 2. SNPs associated with elevated BP and/or HTN

Reference SNP	MAF (1000 Genomes Project)	C-score	Associated Phenotype	Supporting Literature	PMID	TaqMan Assay ID
rs1173756	*t* = 0.3740/1873	0.13	systolic BP	Wain et al.	21909110	C_8809430_1_
rs1173766	*t* = 0.3988/1997	0.06	HTN; 1.16 odds ratio	Kato et al.	21572416	C_27051776_10
rs1173771	A = 0.3391/1698	-0.14	systolic BP	Ehret et al.	21909110,21909115	C_8809478_10
rs1421811	G = 0.2778/1391	0.06	systolic BP	Johnson et al.	22100073	C_8809215_10
rs2270915	G = 0.1955/979	1.90	systolic BP; 4 yr CV mortality 12.5%	Saulnier et al.	21464461	C_15958985_20
rs146301345	A = 0.0008/4	2.55	height	Marouli et al.	28146470	C_171467879_10

CV, cardiovascular; HTN, hypertension; MAF, minor allele frequency; SNP, single nucleotide polymorphism.

Several scoring algorithms (2, 28, 30) were employed to assess functional damage of variants and calculate a C-score (17). Analysis of rs2270915 within the group of HLHS patients from our center mentioned above (some of which also have CoA) revealed a frequency of ~65%, as compared with 14–22% in U.S. populations without CoA. Interestingly, rs146301345 was present in two of these patients studied. These two SNPs are likely to render *NPR-C* nonfunctional, with scoring values indicating they are highly intolerant and damaging (e.g.; rs2270915 C-score = 1.90 and rs146301345 C-score = 2.55). Detection of these two *NPR-C* variants was therefore performed using TaqMan Predesigned SNP Genotyping Assays and buffy coat DNA from 242 total CoA patient samples obtained at the time of corrective surgery. Primers and probes were purchased from Applied Biosystems (Foster City, CA), and probes were labeled with the fluorescent dyes VIC and FAM. Assays were performed using TaqMan Universal Master Mix, with 20 ng of DNA per reaction. The reaction was carried out with a total reaction volume of 5 µl, following an amplification protocol suggested by the manufacturer, with 30 DNA samples run per plate. Genotype was called using the QuantStudio 7 Flex Real-Time PCR System in the presence of a known positive control for each genotype and a no template negative control. Allele and genotype frequencies of *NPR-C* were obtained by direct counting. Comparison of variant frequencies in our CoA patients to the global population was done using the minor allele frequency (MAF) calculation. The global population information was found on NCBI's SNP database (dbSNP) 1000 Genomes project.

Relating NPR-C variants to HTN in CoA patients.

Clinical records were retrospectively analyzed to relate *NPR-C* variants to HTN status using age, sex, height, and follow-up BP. Patients <18 yr at follow-up were evaluated using the NHBPEP Working Group on Children and Adolescents (27a) criteria for systolic and diastolic BP: normal (≤50th percentile BP), pre-HTN (≥50th but <99th percentile BP), HTN (≥99th percentile BP). Patients ≥18 yr at follow-up were evaluated using Joint National Committee seven guidelines for BP: normal (<120/80 mmHg), pre-HTN (120–139/80–89 mmHg), HTN (includes *stage 1*: 140–159/90–99 mmHg and *stage 2*: >160/>100 mmHg). An error-correcting output code for multiclass learning (i.e., machine learning) in MATLAB was proposed with a range of classifiers (e.g., Logistic Regression, Support Vector Machines, Ensemble, etc.) and predictors (e.g., the patient covariates such as age and sex) to determine if either variant alone, in concert with each other and other covariates, is sufficiently predictive of HTN status to be applied with future CoA patients.

Assessing functional significance using cell culture and intact arteries.

Cell culture and ex vivo intact artery approaches were used to assess the potential functional relevance of changes in NPR-C using associated agonists and inhibitors. Human aortic endothelial cells (HAEC) from Cell Biologics (Chicago, IL) were cultured with the manufacturer's recommended media. HAEC were split in a 1:3 ratio every 3–4 days after initial plating from a frozen vial into a T25 cell culture flask. The cells were transferred to six-well plates at *passage 8* and left to grow for 4–5 days to reach confluence. HAEC (n = 4/group) intracellular calcium transients ($[Ca^{2+}]_i$) were recorded during experimentation with a confocal imaging system Leica TCS MP5. Imaging settings were chosen to allow for continuous image capture every 2.3 s and set for a maximum duration of 5 min. After starting acquisition, C-type natriuretic peptide [CNP; induces relaxation via NPR-C (4, 7)] or $cANF^{4–23}$ (specific NPR-C agonist) was diluted into the dish to concentrations of 1.5e-6M and 7.5e-7M, respectively. These doses balanced response to an agent with cellular toxicity. The experiment ended, and the image stack was saved when no additional changes in intensity were observed for 20 s.

Images were quantified using the Loci Tools plugin within ImageJ (National Institute of Health). Briefly, image frames corresponding to times just before drug administration, and the peak response time (for dimming or elimination) were determined. The Measurement option within the Analyze tool was then employed using at least four cells at each time point. Resulting intensities were normalized to background. The EC response to CNP and $cANF^{4–23}$ was also quantified after pretreatment with the NPR-C specific inhibitor, M372049, (AstraZeneca)

(37) for 45 min. Experiments were repeated using four separate plates for each group. Normalized intensities were then averaged, and statistical analysis was performed as described in the myography methods below.

To confirm the HAEC experiments above replicate the in vivo condition, we assessed the endothelial response to CNP under normal deformation and pathologic conditions from CoA with intact aortic sections from the transverse arch of rabbits (*n* = 5) exposed to CoA induced by wire of known diameter (0.051–0.064"). Data from control rabbits (*n* = 3) were also obtained. Specifically, two-photon microscopy was used to visualize and quantify $[Ca^{2+}]_i$ mobilization in response to CNP for endothelial cells from proximal and distal aortic sections of Control and CoA rabbits exposed to CoA by methods described in detail elsewhere (10, 11, 29). Briefly, aortic segments were extracted, opened longitudinally, and loaded with the Ca^{2+} dye, Fluo-4AM (5 μM, Life Technologies), using 0.05% pluronic acid (Pluronic F-127, Sigma-Aldrich) in physiological salt solution (PSS) for 1 h. Aortas were washed and then transferred to a silicone-coated plate containing 2 mM Ca^{2+} buffer, where they were pinned down with the endothelium facing upright. The plate was then transferred to an upright Olympus Fluoview FV1000 microscope (Olympus) equipped with Ti:sapphire lasers tuned to 820 nm and imaged with a 25X (N.A. 1.05 and working distance 2 mm) water-immersion objective lens (XLPL25XWMP, Olympus). The fluorescent response was imaged within EC after addition of 10 μM CNP. Images were taken every 1.6 s. Average Ca^{2+} transients of EC were calculated for proximal and distal sections ($n \geq 9$ cells per location). One rabbit of each group was used to optimize incubation and signal intensity protocols for use with two-photon imaging. Two samples from each vascular region (i.e., proximal and distal) were studied for each rabbit.

The arterial response to potential changes in NPR-C was measured by myography (26). Relaxation was measured for endothelium-intact arterial rings from proximal and distal locations (*n* = 2/location) of the same CoA and Control rabbits used for two-photon microscopy. Relaxation via NPR-C was studied in cumulative doses (10^{-9} to 10^{-6} M) of CNP and atrial natriuretic peptide (ANP), which is also reported to have a strong affinity for NPR-C (23). Aortic segments from the descending thoracic aorta upstream of the coarctation (i.e., proximal) and just downstream of any poststenotic dilatation (i.e., distal) were precontracted with 0.32 μM phenylephrine (PE) before each dose response, and relaxation curves were constructed as percentages of precontracted tension. Care was taken to ensure and present data only from samples with a similar level of tension at the start of each cumulative dose response. Samples were rinsed with fresh PSS three times and allowed to relax back to resting tension after the conclusion of each dose response. In separate trials, arteries were incubated with 10 μM of the NPR-C specific inhibitor (37) M372049 for 30 min. Additional CNP and ANP cumulative dose responses were then conducted in the presence of the inhibitor. Statistical analysis was conducted by one-way multiple analysis of variance (ANOVA). A *P* value <0.05 was considered statistically significant.

RESULTS

Transcriptome analysis.

RNA-Seq of aortic tissue harvested during surgical treatment of human CoA patients revealed a statistically significant reduction in the normalized expression of *NPR-C* for proximal as compared with distal sections (Fig. 2*A*). Relative quantification of *NPR-C* vs. GAPDH from these samples showed a good correlation (R-value of 0.8) with those from qPCR. Rabbit aortic tissue from the proximal region exposed to elevated BP due to the presence of a 20 mmHg coarctation (19) caused a decrease in *NPR-C* intensity in untreated as compared with control rabbits, and this decrease persisted in proximal aortic tissue of rabbits whose coarctation had been relieved by resorption of the dissolvable Vicryl suture used to create the initial constriction.

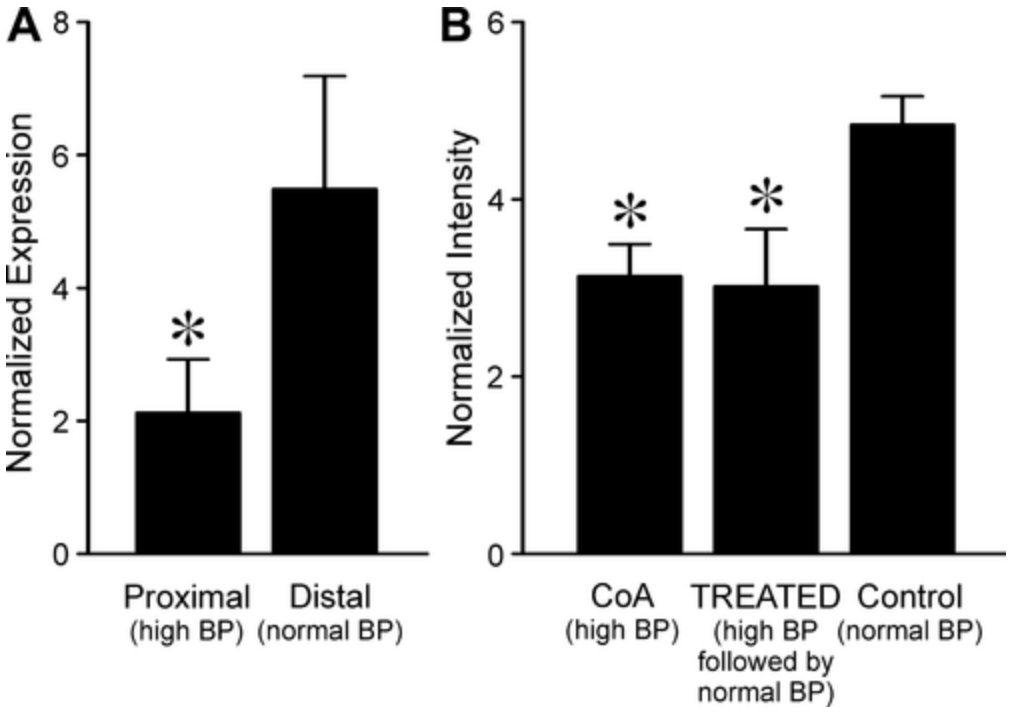

Fig. 2. Normalized natriuretic peptide receptor C (*NPR-C*) expression from RNA sequencing (RNA-Seq) of aortic tissue harvested during surgical treatment of human CoA patients (*A*). All patients had systolic blood pressure (BP) >99% for age, thus underscoring the mechanical impact of CoA. Prior microarray results from a rabbit model of CoA (*B*) also showed a decrease in *NPR-C* transcript levels in proximal aorta exposed to elevated BP from the presence of a coarctation. Data expressed as means ± SE. **P* < 0.05.

Cell culture and intact artery experiments.

HAEC showed rapid decrease in cytosolic $[Ca^{2+}]_i$ response (Fluo-4 AM) in response to 1.5e-6M CNP relative to the baseline period before CNP administration. The NPR-C agonist $cANF^{4-23}$ increased $[Ca^{2+}]_i$ level at a dose of 7.5e-7M. Both responses were blocked by the NPR-C inhibitor M372049 (Fig. 3) when evaluated at the same respective time durations following respective substance administration.

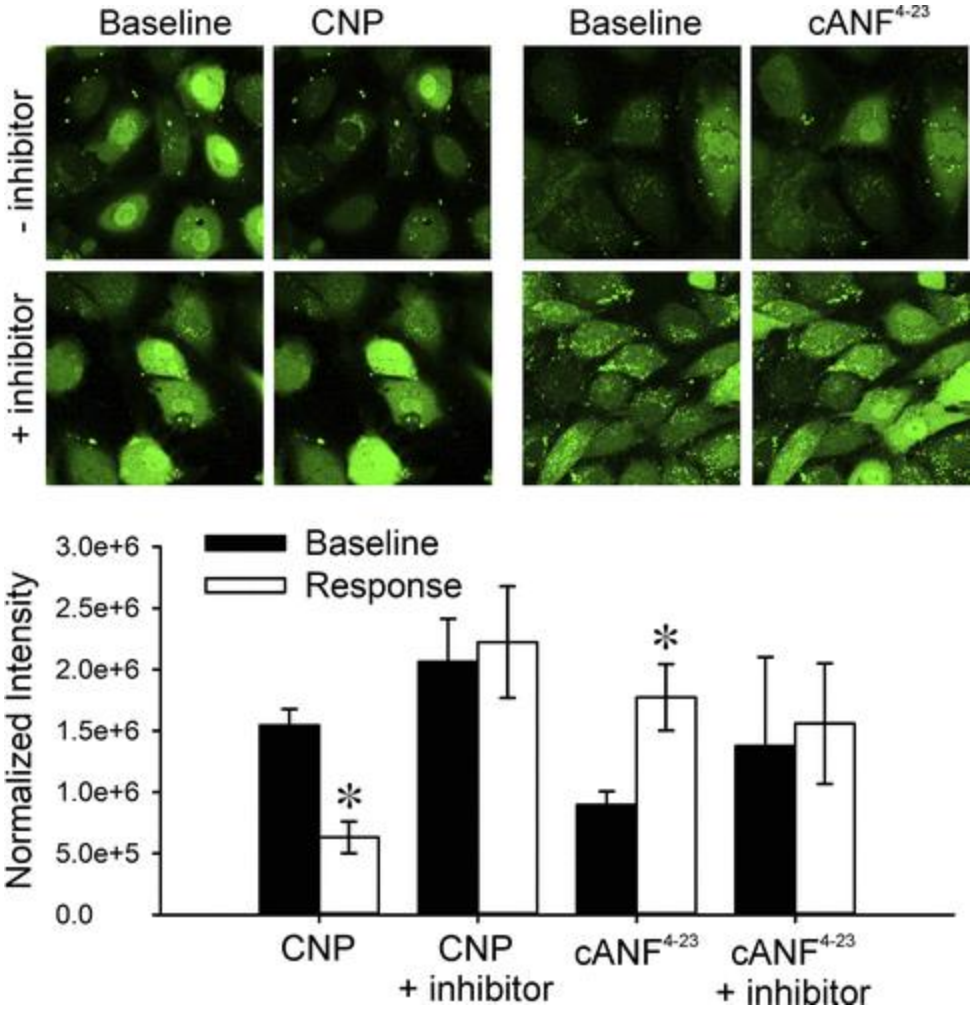

Fig. 3. Human aortic endothelial cells (HAEC) showed decreased intracellular calcium ($[Ca^{2+}]_i$) response to C-type natriuretic peptide (CNP, 1.5e-6M). The NPR-C agonist $cANF^{4–23}$ (7.5e-7M) increased $[Ca^{2+}]_i$ release. Both responses were blocked by the NPR-C inhibitor M372049. *$P < 0.05$ vs. Baseline.

The average catheter-based BP gradient measured in CoA rabbits of the current study was 17 mmHg, while that measured from spatially equivalent locations of Control rabbits was 1 mmHg. Similar to HAEC, two-photon imaging of EC within intact aortic segments from proximal and distal locations of Control rabbits revealed a decrease in $[Ca^{2+}]_i$ in response to CNP (Fig. 4). This decrease in was also observed for intact aortic segments from the distal aorta of CoA rabbits where there was normal BP in vivo. In contrast, for CoA rabbits the $[Ca^{2+}]_i$ response to CNP from proximal aorta segments experiencing high BP in vivo was significantly attenuated when compared with the distal segments (Fig. 4).

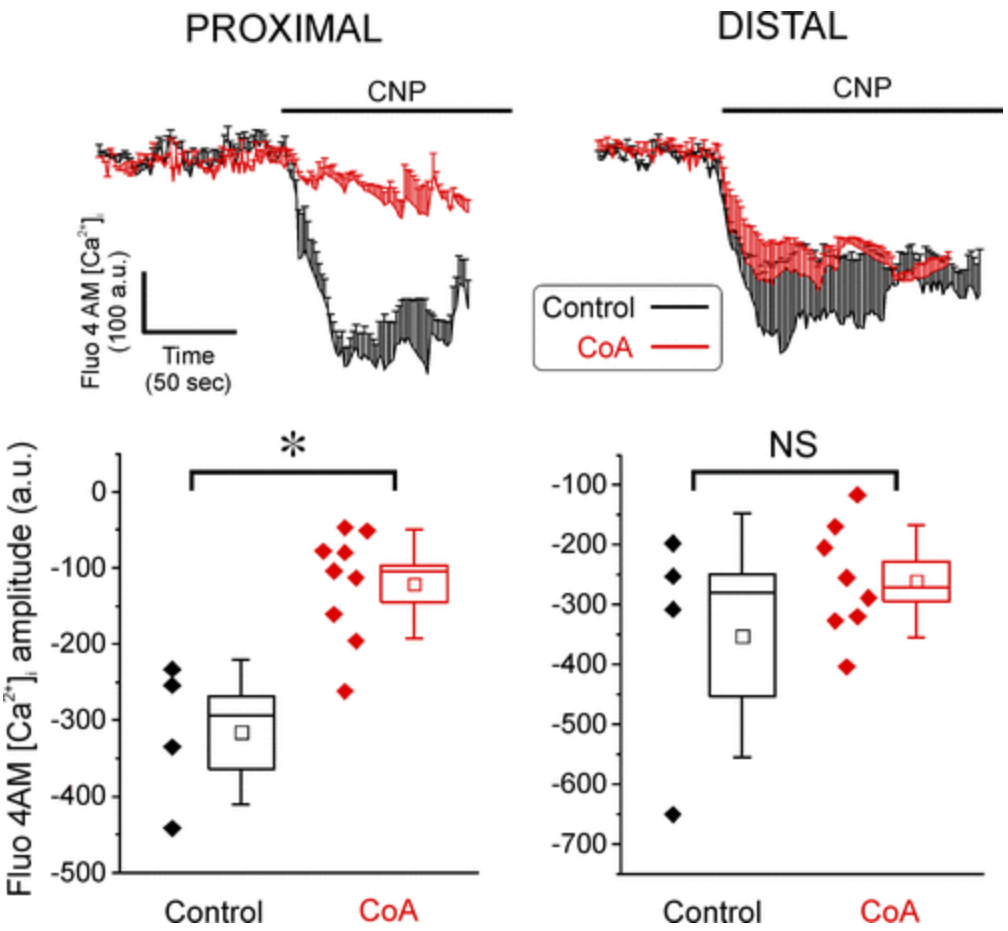

Fig. 4. Two-photon images of $[Ca^{2+}]_i$ levels from endothelial cells (EC) within intact sections for groups of Control and CoA rabbits at baseline and upon administering CNP (10 μM). Temporal results show differences in proximal vs. distal locations (*n* = 9 EC/location). CNP decreased $[Ca^{2+}]_i$ response for ECs within both sections of Control rabbits and distal sections of CoA rabbits, but this response is absent in proximal sections of CoA rabbit, which were exposed to high BP in vivo.

Figure 5 shows active relaxation of the aorta to CNP and ANP from CoA and Control rabbits. There were no differences in relaxation of proximal vs. distal aortic rings from Control rabbits when exposed to increasing doses of CNP or ANP (Fig. 5, *top* row). In contrast, there was a statistically significant attenuation of relaxation in proximal as compared with distal aortic rings for CoA rabbits when exposed to all doses of CNP and ANP (Fig. 5, *middle* row). ANP-induced relaxation of aortic rings from proximal and distal locations was nearly twice that observed with CNP. Dose-dependent relaxation to CNP and ANP was impaired and not statistically different between proximal and distal aortic rings following pretreatment with the NPR-C inhibitor M372049 (Fig. 5, *bottom* row).

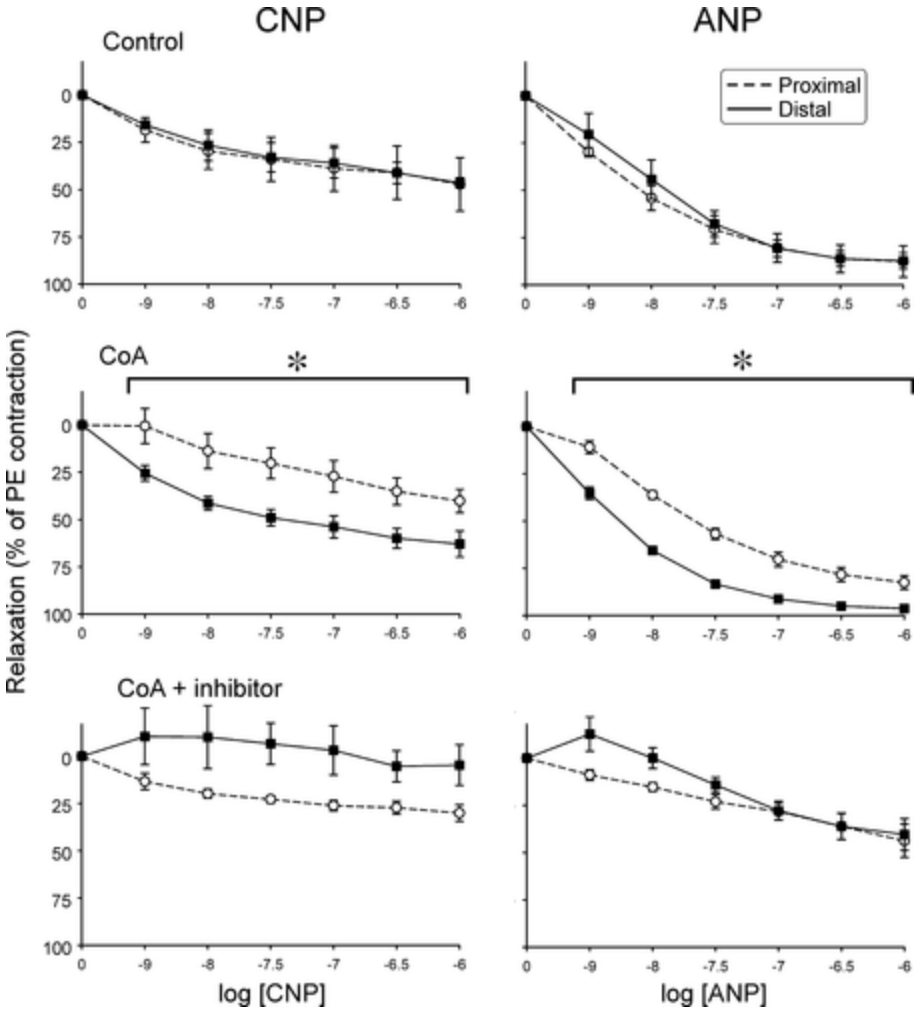

Fig. 5. Dose-dependent relaxation of the proximal and distal aorta to CNP (*left*) and atrial natriuretic peptide (ANP, *right*) for groups of Control and CoA rabbits. Relaxation of proximal and distal aortic rings was not different in samples from Control rabbits (*top* row). There was statistically significant attenuation of relaxation in proximal as compared with distal aortic rings for CoA rabbits when exposed to all doses of CNP and ANP (*middle* row). Residual relaxation to these peptides was abolished by pretreatment with the NPR-C inhibitor M372049 (*bottom* row). PE, phenylephrine.

Genotyping of NPR-C variants.

DNA samples of patients from our center were genotyped to determine if HTN in CoA patients may be associated with the *NPR-C* variants rs2270915 and rs146301345, which are predicted to be highly intolerant and damaging. Figure 6 shows allelic discrimination plots from samples on representative plates for each variant. Figure 6*, left*, reveals the majority of CoA patients have an rs2270915 genotype of AA, while AG is also common. We therefore focused on samples from CoA patients that were homozygous for the opposite allele (GG; within the ellipse). Figure 6, *right*, shows samples for rs146301345 that are homozygous for the VIC probe, which corresponds to a genotype of GG. This is the common genotype in the human population, therefore focusing our interest to samples that were heterozygous, as shown by those within the ellipse. The frequencies of *NPR-C* variant genotypes in our CoA cohort from all plates are summarized in Table 3.

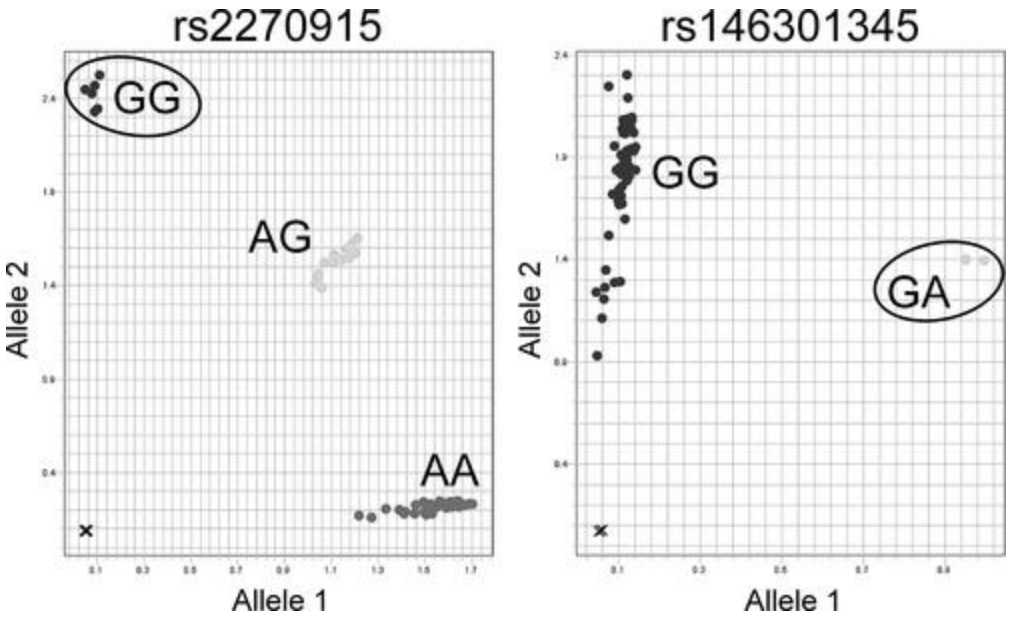

Fig. 6. Allelic discrimination plots for a representative plate from each *NPR-C* variant. Samples with the noncommon genotype for each variant are indicated by the ellipses.

Table 3. Frequency of NPR-C variant genotypes in the current cohort of CoA patients

	Genotype	Patients, *n*	Percentage
rs2270915	A/A	155	64.1
	G/A	71	29.3
	G/A	16	6.61
46301345	A/A	0	0
	A/G	5	2.07
	G/G	237	97.9

For rs2270915, the minor allele is G, which has a frequency of 19.6% in the global population, having been observed 979 times in the sample population of 2,500 people (or 5,000 chromosomes). Similarly, our current cohort of CoA patients revealed a frequency for rs2270915 of 21.3%. The minor allele for rs2270915 was detected 103 times in 242 patient samples (484 chromosomes). The minor allele for rs146301345 is A. This variant is more rare in the global population with a frequency of only 0.08% (4 of 2,500 people, or 5,000 chromosomes). In contrast, rs146301345 was significantly enriched in our cohort of CoA patients having been observed five times in just 242 people (or 484 chromosomes).
Unfortunately, rs2270915 and rs146301345 alone, or together, did not have sufficient predictability to determine HTN status in our cohort. Calculation of sample size using the descriptive statistics above for these variants in our CoA cohort indicates ~2,500 patients must be studied to determine the ability of rs146301345 to predict hypertensive status.

DISCUSSION

For unknown reasons, treated CoA patients often have a reduced life expectancy from CV morbidity, primarily from HTN (8). Upon closer review, there are several potential contributors to arterial pathology in CoA. Putatively, there is a causal genetic basis for the initial narrowing. The prevailing causal hypothesis is based on histology showing that tissue with features similar to the ductus arteriosus (DA) also exists near the coarctation, suggesting CoA may be created during closure of the DA in the first week of life (9, 16). Closure of the DA leads

to the secondary consequence of exposing the aortic arch and arteries above the coarctation to mechanical stimuli including elevated BP and a gradient across the narrowing. Treatment approaches that mitigate the secondary consequence of CoA may ultimately reduce morbidity for a large number of CoA patients and individuals with refractory HTN attributable to prolonged mechanical stimuli.

The critical barriers to identifying the mechanisms of morbidity in humans with CoA from causal or mechanical sources include a relatively small number of heterogeneous patients at each center. To circumvent these barriers the current investigation analyzed gene expression data from an animal model of CoA largely devoid of the heterogeneity seen clinically (26) based on RNA-Seq results of human aortic tissue harvested during surgical treatment. The goal of this approach was to identify a candidate gene that could be further studied in the rabbit, but that was motivated from humans with CoA who had upper extremity systolic BP >99th percentile for their sex, height, and age, thereby focusing on mechanical consequences. The primary finding of the current investigation is that RNA-Seq from humans treated for CoA revealed downregulation of *NPR-C* in proximal sections of the thoracic aorta subjected to high BP when compared with sections from regions in the distal thoracic aorta exposed to normal BP. Importantly, microarray data from our experimental rabbit model of CoA also showed downregulation of *NPR-C* in proximal aortas from both CoA and treated rabbits experiencing high BP, as compared with controls experiencing normal BP (Fig. 2).

NPR-C is a single transmembrane receptor coupled to the activation of phospholipase C beta-3 (27) through beta-gamma subunits and adenylyl cyclase inhibition via inhibitory guanine nucleotide regulatory protein (Gi) (3). NPR-C is found in many tissues including SMC where it has been shown to play a role inhibiting proliferation (5, 15), and endothelial cells where recent literature suggests a role in re-endothelialization and viability under healthy conditions (15). Prior studies also point to a role in regulating BP (3) as systemic administration of an NPR-C agonist attenuated high BP in spontaneously hypertensive rats by inhibiting enhanced levels of Gi (21). NPR-C is one of three receptors for natriuretic peptides that include ANP, brain (BNP) and CNP. ANP and BNP are mostly found in the atria and ventricles, whereas CNP is abundantly expressed in vascular EC (4, 7). Activation of NPR-C increases eNOS activity resulting in the formation of NO and vasorelaxation via cGMP. While this prior literature implicates NPR-C in arterial dysfunction, the current investigation is the first to show altered *NPR-C* transcript levels in aortic tissue from human CoA patients.

Similarity in trends between the current human RNA-Seq results and prior microarray results from rabbits with CoA suggest that the rabbit provides a unique model to further unravel the contribution of NPR-C to CoA-induced arterial dysfunction. Indeed, the data in Figs. 4 and 5 show the functional relevance of NPR-C impairment in the vasculature using this model and are consistent with the cell culture results in Fig. 3. Collectively these data provide the first exciting evidence of a functional relevance for changes in NPR-C from CoA.

The current results show promising evidence of functional ramifications to altered NPR-C activity from pronounced mechanical stimuli. Interestingly, genome-wide association studies (GWAS) have uncovered several known SNPs for *NPR-C* that are associated with high systolic BP or HTN (Table 2). For example, rs2270915 presents in 14–22% of U.S. populations (1000 Genomes Project), with the altered genotype having higher systolic BP and sodium response (32). While our rabbit model of surgical coarctation induction certainly suggests decreased *NPR-C* transcript levels are a secondary consequence of mechanical stimuli, aortic tissue from humans with CoA that was used for RNA-Seq may theoretically include changes in gene expression from causal as well as mechanical sources. We attempted to mitigate this issue by using tissue from patients who had upper extremity systolic BP >99th percentile for their sex, height, and age, thereby focusing on the mechanical consequences of the coarctation. Nevertheless, an alternative hypothesis is that normotensive CoA patients may simply have a normal *NPR-C* genotype. The ability to genotype CoA patients for *NPR-C* variants is attractive as a potential screening tool, as it could explain the presence of HTN even in CoA patients who have

undergone optimal treatment with no residual blood pressure gradient. While the MAF for rs2270915 in the current CoA cohort was not different from the general population, the more rare SNP, rs146301345, was enriched in our cohort of coarctation patients. This finding suggests that in addition to mediating coarctation-induced arterial dysfunction from mechanical stimuli, NPR-C may in some way be causal for HTN in CoA. The full understanding of this finding is not currently known and may elude researchers for some time as our sample size estimates from power analyses indicate ~2,500 CoA patients will be needed to statistically determine whether rs146301345 is associated with HTN. This represents a large number as most centers will see ~20 new CoA patients annually. Moreover, updated guidelines for the prevention, detection, evaluation, and management of BP were recent released, which includes BP categories that differ slightly from those used in the current study (38). Future studies using the updated guideline and categories will be needed to determine potential association with HTN. Additional studies will also be needed to better understand the precise mechanism(s) by which *NPR-C* variants seen clinically result in HTN via markers of endothelial function (e.g., NO release).

The current results should be interpreted relative to several potential limitations. Healthy aortic tissue was not available from an age- and sex-matched cohort of subjects. We therefore used distal aortic tissue we know to be exposed to a normal range of BP (20, 25), even though other mechanical stimuli such as wall shear stress may impact gene expression downstream of the coarctation. We acknowledge that different approaches were used to quantify changes in gene expression between humans and rabbits exposed to CoA. This was unfortunate but is an unavoidable consequence resulting from the continuing evolution of genomic tools. To mitigate differences, DEG from completion of the data analysis process applied for each respective approach (RNA-Seq vs. microarray) were compared with identified candidate genes. Moreover, the data featured here in support of a functional role for altered NPR-C activity, and that rs146301345 seems to be enriched in our coarctation patients, suggest that NPR-C is indeed a target of relevance to CoA. In contrast to finding new rare SNPs, we focused on SNPs known to be associated with high BP given the prevalence of HTN in CoA (32.5%, range 25–68%) (6). Moreover, uncovering new variants from the population of CoA patients at one center is difficult due to a limited sample size. Two-photon imaging is best performed on fresh tissue sections, and the extended duration of associated imaging sessions following tissue harvest precluded the additional study of responses to ANP via two-photon imaging as was performed via myography.

In conclusion, the current investigation identified NPR-C as a potential contributor to arterial dysfunction in CoA patients for the first time. Specifically, RNA-Seq of tissue from humans with CoA showed downregulation of *NPR-C* in proximal aortic sections exposed to elevated BP, as compared with distal sections from normotensive regions. Complementary microarray data from an experimental rabbit model of CoA similarly showed downregulation of *NPR-C* in proximal aortas from both CoA and treated rabbits experiencing high BP as compared with control rabbits experiencing normal BP. Intact artery segments from this experimental model were used with HAEC to obtain data on the functional ramifications of altered NPR-C activity. Both approaches showed decreased $[Ca^{2+}]_i$ activity in response to CNP administration under normal conditions. The normal response to CNP and ANP is abolished for aortic segments previously exposed to elevated BP from CoA. Moreover, NPR-C could represent a potential therapeutic target as pharmacological inhibition resulted in impaired aortic relaxation. Of the known *NPR-C*variants predicted to be highly intolerant and damaging, rs146301345 was found to be enriched in our cohort of CoA patients, but a small samples size precluded determination of its potential association with HTN in CoA patients. These results may ultimately be used to suggest a new paradigm for CoA treatment based on mechanical stimuli, genotyping, and/or changes in arterial function assessed clinically after confirmation of the current important findings in larger studies.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

J.F.L., A.T.-M., K.B., A.P.Z., M.M.K., and T.J.E. conceived and designed research; J.F.L., K.B., D.K.M., B.J.W., J.W.G., K.R., O.P., A.P.Z., and M.E.M. performed experiments; J.F.L., K.D.S., K.B., D.K.M., M.A.G., B.J.W., J.W.G., K.R., O.P., A.P.Z., T.J.E., A.A.A., and M.E.M. analyzed data; J.F.L., A.T.-M., K.D.S., D.K.M., M.A.G., B.J.W., K.R., O.P., A.P.Z., M.M.K., T.J.E., A.A.A., and M.E.M. interpreted results of experiments; J.F.L., B.J.W., K.R., O.P., A.P.Z., and M.E.M. prepared figures; J.F.L., A.T.-M., T.J.E., and A.A.A. drafted manuscript; J.F.L., A.T.-M., K.D.S., K.B., D.K.M., M.A.G., B.J.W., J.W.G., K.R., O.P., A.P.Z., M.M.K., T.J.E., A.A.A., and M.E.M. edited and revised manuscript; J.F.L., K.D.S., K.B., D.K.M., M.A.G., B.J.W., J.W.G., K.R., O.P., A.P.Z., M.M.K., T.J.E., A.A.A., and M.E.M. approved final version of manuscript.

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