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ORIGINAL RESEARCH

Natriuretic Peptide Receptor 2 Locus Contributes to Carotid Remodeling

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BACKGROUND: Carotid artery intima/media thickness (IMT) is a hallmark trait associated with future cardiovascular events. The goal of this study was to map new genes that regulate carotid IMT by genome-wide association.

METHODS AND RESULTS: We induced IMT by ligation procedure of the left carotid artery in 30 inbred mouse strains. Histologic reconstruction revealed significant variation in left carotid artery intima, media, adventitia, external elastic lamina volumes, intima-to-media ratio, and (intima+media)/external elastic lamina percent ratio in inbred mice. The carotid remodeling trait was regulated by distinct genomic signatures with a dozen common single-nucleotide polymorphisms associated with left carotid artery intima volume, intima-to-media ratio, and (intima+media)/external elastic lamina percent ratio. Among genetic loci on mouse chromosomes 1, 4, and 12, there was natriuretic peptide receptor 2 (*Npr2*), a strong candidate gene. We observed that only male, not female, mice heterozygous for a targeted *Npr2* deletion ($Npr2^{+/-}$) exhibited defective carotid artery remodeling compared with *Npr2* wild-type ($Npr2^{+/+}$) littermates. Fibrosis in carotid IMT was significantly increased in $Npr2^{+/-}$ males compared with $Npr2^{+/-}$ females or $Npr2^{+/+}$ mice. We also detected decreased *Npr2* expression in human atherosclerotic plaques, similar to that seen in studies in $Npr2^{+/-}$ mice.

CONCLUSIONS: We found that components of carotid IMT were regulated by distinct genetic factors. We also showed a critical role for *Npr2* in genetic regulation of vascular fibrosis associated with defective carotid remodeling.

Key Words: carotid artery ■ genome-wide association ■ inbred mice ■ *Npr2* ■ vascular remodeling

Atherosclerosis is a complex disorder regulated by multiple genetic and environmental factors.[1] Recently, many candidate genetic mechanisms have been proposed but most do not have major effects on development of human atherosclerosis.[2,3] A reliable clinical measure of atherosclerosis progression is carotid intima/media thickness (IMT), which predicts cardiovascular complications.[4] Genetic linkage studies in Framingham Heart and Dominican Family cohorts identified significant quantitative traits loci (QTLs) on human chromosome (chr)7q, chr12q, and chr14q that control carotid IMT.[5,6] Genome-wide association (GWA) studies mapped a number of single-nucleotide polymorphisms (SNPs) associated with variation in carotid IMT.[2,3,7] Despite such advances, those studies were underpowered and there are technical limitations to assessing specific mechanisms of carotid IMT and atherosclerotic plaque progression and regression in humans.[8–10]

We developed a robust mouse model of carotid IMT and showed significant genetic effects in 5 inbred strains of mice.[11,12] A forward genetic approach followed by congenic mapping was effective in uncovering 3 carotid intima modifier QTLs on mouse chr2, chr11, and chr18.[13–15] Additional QTLs were identified on mouse chr5, chr9, chr12, and chr13, that contribute

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CLINICAL PERSPECTIVE

What Is New?

- Genetic variation in natriuretic peptide receptor 2 (*Npr2*) associated with defective carotid artery remodeling in a panel of mice that represent a human population.
- Male but not female mice with a targeted *Npr2* depletion exhibited defective carotid artery remodeling with increased fibrosis.
- Expression of the *Npr2* was reduced in human atherosclerotic plaques.

What Are the Clinical Implications?

- *Npr2* is a plausible target for new diagnostic and therapeutic approaches to treat vascular disease.

Nonstandard Abbreviations and Acronyms

3D	3-dimensional
BP	systolic blood pressure
chr	human chromosome
EEL	external elastic lamina
eNOS	endothelial nitric oxide synthase
IMT	intima/media thickness
LCA	left carotid artery
***Npr2*$^{-/-}$**	Npr2 knockout
Npr2	natriuretic peptide receptor 2
***Npr2*$^{+/-}$**	Npr2 heterozygous
***Npr2*$^{+/+}$**	Npr2 wild-type
QTLs	quantitative traits loci
SNP	single-nucleotide polymorphism

to carotid atherosclerosis.[16–18] A plausible approach to identification of atherosclerosis traits includes GWA studies in a panel of mouse inbred strains.[19] In an earlier study, we utilized this approach and identified several causative genes that control the elevated heart rate trait.[20] The primary goal of this study was to use GWA to identify new genes that regulate carotid remodeling. By studying 30 strains of mice, we characterized a genetic locus that houses a novel gene candidate—natriuretic peptide receptor 2 (*Npr2*)—that regulates carotid artery remodeling.

METHODS

The authors declare that all supporting data are available within the article and its online supplementary material.

Animals

We studied flow-induced carotid remodeling in 9- to 12-week-old male mice from 30 inbred strains (Table S1). We purchased 10 mice per strain, 4 sham and 6 ligated, from the Jackson Laboratory (Bar Harbor, Maine). However, some of the inbred mouse strains exhibited poor survival and required additional mice, as we recently reported for 129X1 in a comparison with C57BL/6J.[21] We also used male and female *Npr2* wild-type (*Npr2*$^{+/+}$) and *Npr2* heterozygous (*Npr2*$^{+/-}$) littermate mice from our recently established colony.[22] Presence of *Npr2* alleles was determined by genotyping, as described previously.[23] We were unable to use *Npr2* knockout (*Npr2*$^{-/-}$) mice for surgery because of low body weights.[23] Experimental mice were housed individually under a 12-hour light/12-hour dark cycle with free access to water and chow. All animal procedures were approved by the animal care committee of the University of Rochester and were in accordance with the *Guide for the Care and Use of Laboratory Animals*.[24]

Tail-Cuff Plethysmography

Systolic blood pressure (BP) and heart rate were collected with a BP-2000 (Visitech Systems, Apex, North Carolina) system in *Npr2* mice. We performed our experiments according to a method described previously by our group.[11,20,25]

Carotid Artery Ligation

We studied flow-induced carotid remodeling 2 weeks after the ligation procedure in all mice, as described previously.[11] Briefly, animals were anesthetized with a cocktail of ketamine and xylazine (130 and 9 mg/kg, respectively, intraperitoneally). The neck area was opened by a midline incision and the bifurcation of the left carotid artery (LCA) was isolated. The internal and external LCA branches were ligated with 6-0 silk, leaving the occipital artery intact. The neck opening was closed with a 6-0 coated Vicryl suture. An analgesic flunixin meglumine (120 mg/kg, intraperitoneally) was given immediately after and once per day for 3 days after the surgery. A plastic box with additional bedding material was given to mice housed individually after the surgery.

Vascular Ultrasound

We measured blood flow in the LCA in inbred mouse strains with a ultrasonic transit-time volume flowmeter (Transonic Systems, Ithaca, New York) before termination, as described elsewhere.[11] Carotid artery imaging in anesthetized *Npr2* mice was done with a Vevo2100 machine (FUJIFILM VisualSonics, Toronto, Ontario, Canada) as described in our previous work.[26]

Histology and Morphometry

Two weeks after ligation, mice were perfusion fixed under anesthesia; carotids were collected, processed, and stained with hematoxylin and eosin; followed by morphometry analyses (MCID image software), as described elsewhere.[11] We evaluated 10 area divisions of the LCA from the bifurcation every 200 μm through the 2-mm length. Averaged area measurements of the LCA for each group were used to produce 3-dimensional (3D) images with MATLAB programming (The Mathworks, Natick, Massachusetts), as described elsewhere.[26] Unstained LCA sections of ligated *Npr2* mice were processed with an Alcian Blue kit (ScienCell Research Laboratories, Carlsbad, California) or PicroSirius Red kit (Abcam, Cambridge, Massachusetts). We also stained consecutive cross-sections of human endarterectomy with rabbit anti-NPR2 (1:100; overnight at +4°C; Abcam), mouse anti-human α_1 smooth muscle actin (1:1000; 60 minutes at room temperature; hematoxylin and eosin stain), or mouse anti-human CD68 (1:1000; 60 minutes at room temperature; eBiosciences, Thermo Fisher Scientific, Waltham, Massachusetts) antibodies followed by a secondary goat anti-rabbit (1:400; Vector Laboratories, Burlingame, California) or horse anti-mouse (1:200; Vector) antibodies with an ABC kit (Vector) and counterstained with hematoxylin, as described previously.[21,22] A percentage of positive staining was determined in stained mouse and human sections by the ImagePro analyzer version 6.2 (Media Cybernetics, Bethesda, Maryland) in a blinded manner as we have reported previously.[27]

Genome-Wide Association of Carotid Remodeling in 30 Inbred Strains of Mice

GWA mapping for carotid remodeling traits was done using an efficient mixed-model association method with a significance for *P*-value thresholds (4.1×10^{-6}) on the basis of power calculations in a similar number of mouse strains.[28] The GWA results on variation for LCA intima, media, adventitia, and external elastic lamina (EEL) volumes, intima/media ratio, and (intima+media)/EEL×100% are listed in Tables S2 through S7. We used the publicly available Integrative Genomic Viewer version 2.4.17 (Broad Institute and Regents of the University of California) to visualize the significant peaks on the mouse genome.[29]

Human Samples

Cross-sections of de-identified human endarterectomy samples from 3 patients undergoing surgery on a carotid artery were collected with the approval from the subjects review board of the University of Rochester School of Medicine and Dentistry Research (RSRB00069961).

Statistical Analysis

Results are presented as mean±SEM. Statistical significance was determined using JMP version 13.0.0 software (SAS). Initial analyses of data sets across experiments showed a normal distribution. Two groups were compared using Student's *t* test. One-way ANOVA was evaluated for each parameter with post-hoc comparisons of means using the Tukey–Kramer honestly significant difference test. We performed multivariate and linear regression analyses between LCA intima volumes and LCA (intima+media)/EEL×100% to determine pairwise correlations in the experimental groups. $P<0.05$ was considered significant.

RESULTS

Variation of the Remodeling Traits Across 30 Inbred Mouse Strains

A forward genetic approach followed by congenic mapping was effective in revealing the causes of increased LCA intima.[13–15] To map the carotid intima trait, we investigated variation in the most common inbred strains (Figure 1A). We found that relative changes in LCA blood flow were similar among the studied strains compared with RCA blood flow after ligation (Table S1). However, the same reduction in blood flow resulted in significant variation in carotid arteries on the basis of 3D reconstruction of histologic measurements across the strains (Figures S1 through S3). There were 5 inbred strains that significantly differed in LCA intima volume from controls or other strains, whereas no intima was detected in controls (Figure 1A). Similar results were observed for intima/media ratio and (intima+media)/EEL×100% across 30 mouse strains (Figure 1B and 1C). We observed greater variation in LCA media, EEL, and adventitia volumes (Figures S3 through S6). There was disparity in the relationship between LCA intima volume and (intima+media)/EEL×100%. For example, below 40% LCA (intima+media)/EEL×100% was observed in mouse strains without (eg, BTBR) or with (eg, C3H) intima (Figure 1D). In contrast, mouse strains with a greater percentage of LCA (intima+media)/EELx 100% had small (SM) vs large (SJL) LCA intima volume (Figure 1E). Thus, we characterized a significant variation in response to vascular injury across 30 inbred mouse strains permitting GWA studies for carotid remodeling traits.

GWA Analysis of Carotid Remodeling Traits in 30 Inbred Strains of Mice

Most SNPs were associated with LCA intima/media ratio after ligation (Figure 2B and Table S6). Importantly, there were common SNPs regulating LCA intima volume, intima/media ratio, and

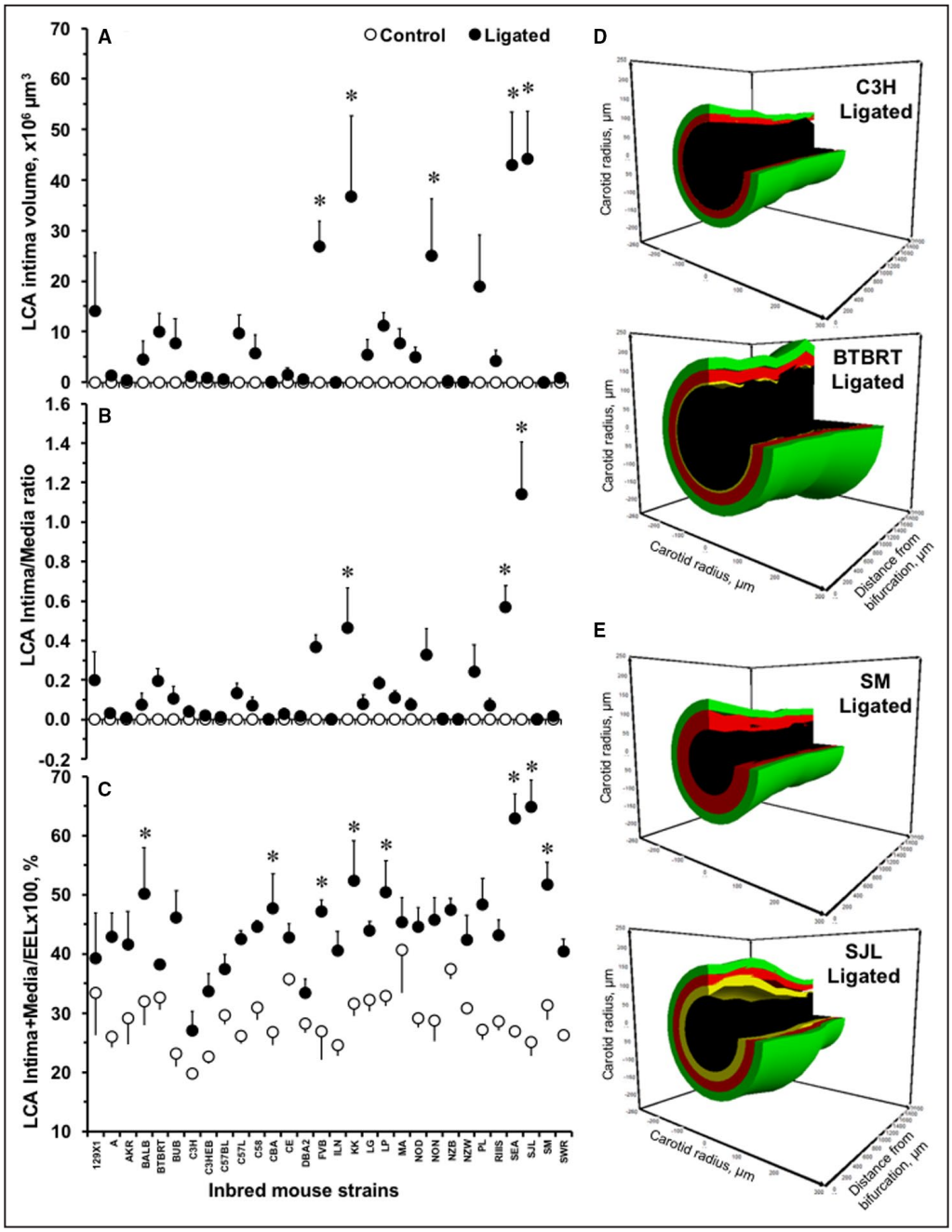

Figure 1. Variation in carotid remodeling in 30 mouse strains.
A, Left carotid artery (LCA) intima volume, $\times 10^{-6}$ μm^3. **B**, LCA intima/media ratio. **C**, LCA (intima+media)/EEL×100%. Open circles indicate controls. Black circles indicate ligated mice. Values are mean±SEM; *P<0.05 vs control or other mouse strains. n=4 to 6 per group. **D**, Representative 3-dimensional (3D) reconstructions of the 2 mm-length from the bifurcation of the ligated LCA in mice with low values of LCA (intima+media)/EEL×100%. **E**, Representative 3D reconstructions of mouse strains with a greater percentage of LCA (intima+media)/EEL×100%. Black color shows lumen, yellow indicates intima, red indicates media, and green indicates adventitia volume. EEL indicates external elastic lamina.

(intima+media)/EEL×100% within genomic locations on mouse chr1, chr4, and chr12 (Figure 2, and Tables S2, S6, and S7). Among 12 common SNPs, we discovered 6 candidate genes (*Slc24a4*, solute carrier family 24 sodium/potassium/calcium exchanger, member 4; *Tln1*, talin 1; *Npr2*, natriuretic peptide receptor 2; *Fam221b*, family with sequence similarity 221, member B; *Tmem8b*, transmembrane protein 8B; *Spaar*, small regulatory polypeptide of amino acid response) known to regulate carotid intima and remodeling (Table). Our findings are supported by another mouse genetic study that proposed *Npr2*

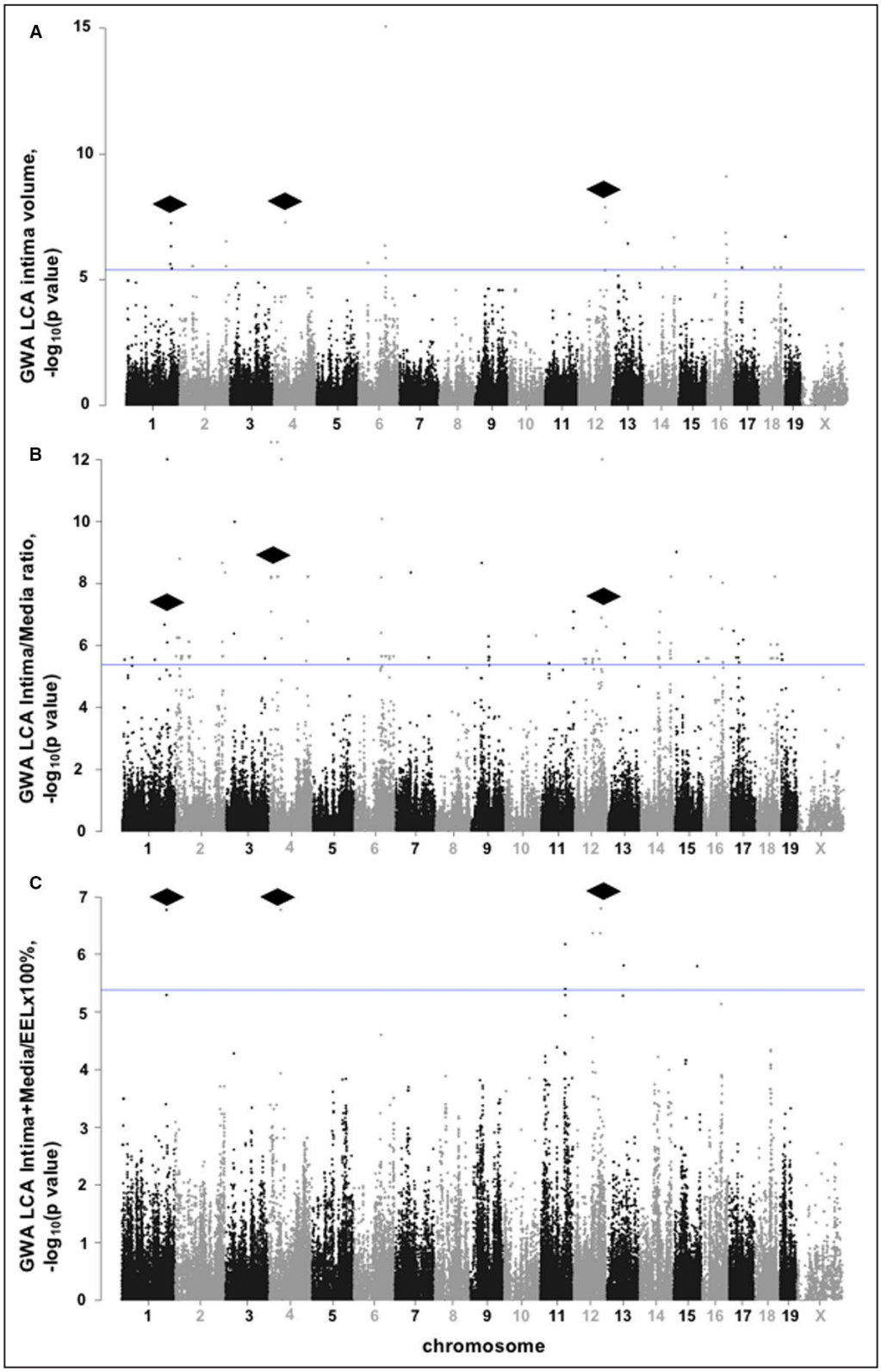

Figure 2. GWA of carotid remodeling traits in 30 mouse strains.
A, GWA of LCA intima volume, $\times 10^{-6}$ μm^3. **B**, GWA of LCA intima/media ratio. **C**, GWA of LCA (intima+media)/EEL×100%. Each circle represents an SNP. Mouse chromosomes are presented on the X-axis. Blue lines show significance threshold. Black rhombi point to common regions of significant SNPs associated with LCA intima, intima/media ratio, and (intima+media)/EEL×100% traits in 30 inbred mouse strains. EEL indicates external elastic lamina; GWA, genome-wide association; LCA, left carotid artery; and SNP, single-nucleotide polymorphism.

Table. Common SNPs Associated With LCA Intima, Intima-to-Media Ratio and (Intima+Media)/EEL×100% Traits in 30 Inbred Mouse Strains

dbSNP	Position, bp	*P* Value	Effect Size	Gene
rs36294984	Chr12: 102175044	1.64E-07	5.35241	*Slc24a4*: intron
rs36281276	Chr12: 102181524	1.64E-07	5.35241	*Slc24a4*: intron
rs36677986	Chr1: 165121248	1.68E-07	5.35456	Intergenic variant
rs37370522	Chr1: 165124350	1.68E-07	5.35456	Intergenic variant
rs38372684	Chr1: 165124376	1.68E-07	5.35456	Intergenic variant
rs27831183	Chr4: 43542462	1.72E-07	5.35142	*Tln1*: intron
rs28320686	Chr4: 43620641	1.72E-07	5.35142	Intergenic variant
rs28320653	Chr4: 43645470	1.72E-07	5.35142	*Npr2*: intron
rs28320604	Chr4: 43650523	1.72E-07	5.35142	*Npr2*: intron
rs28320543	Chr4: 43662649	1.72E-07	5.35142	*Fam221b*: intron
rs28320511	Chr4: 43688855	1.72E-07	5.35142	*Tmem8b*: intron
rs28311534	Chr4: 43732460	1.72E-07	5.35142	*Spaar*: noncoding RNA

bp indicates base pair; EEL, external elastic lamina; *Fam221b*, family with sequence similarity 221, member B; LCA, left carotid artery; *Npr2*, natriuretic peptide receptor 2; *Slc24a4*, solute carrier family 24 (sodium/potassium/calcium exchanger), member 4; SNP, SNP single-nucleotide polymorphism; *Spaar*, small regulatory polypeptide of amino acid response; *Tln1*, talin 1; and *Tmem8b*, transmembrane protein 8B.

with its ligand, C-type natriuretic peptide, as candidate genes in high blood pressure.[30,31] Thus, we found that *Npr2* is a plausible candidate for regulation of carotid remodeling traits in response to injury.

Carotid Remodeling in *Npr2*-*Npr2*$^{+/-}$ Mice

We confirmed earlier genetic studies in mice on a causal role for the *Npr2* gene in salt-induced kidney injury.[22] As we reported for direct BP measurements, tail-cuff systolic BP (114–117 mm Hg) and heart rate (574–618 beats/min) profiles were similar between *Npr2*$^{+/+}$ and *Npr2*$^{+/-}$ males and females (not shown). We found that a carotid ligation procedure resulted in a similar reduction of blood flow (Table S8) or estimated shear stress (not shown) between *Npr2*$^{+/+}$ and *Npr2*$^{+/-}$ littermates compared with sham animals. However, there were significant differences in LCA remodeling on the basis of histologic evaluation across *Npr2* genotypes (Figure 3). We observed a significant increase in LCA intima/media ratio, adventitia, and EEL volumes versus sham animals in male *Npr2*$^{+/+}$ mice (Figure 3A and 3C). In contrast, LCA compartmental volumes were significantly reduced in *Npr2*$^{+/-}$ versus *Npr2*$^{+/+}$ male mice after ligation (Figure 3A and 3C). Histologic evaluation of the LCA revealed sex-dependent differences in response to ligation in *Npr2* genotypes (Figure 3A and 3B). LCA intima/media ratio, adventitia, and EEL volumes were similar among *Npr2*$^{+/+}$ and *Npr2*$^{+/-}$ females (Figure 3B and 3D). In *Npr2*$^{+/+}$ females, we observed lower LCA intima, adventitia, and EEL volumes compared with *Npr2*$^{+/+}$ males (Figure 3C and 3D). Unlike *Npr2*$^{+/-}$ males, we found that *Npr2*$^{+/-}$ females exhibited significantly increased LCA EEL after ligation (Figure 3). Thus, we confirmed that the *Npr2* gene plays a role in carotid artery response to injury, but only in males.

Relationship Between Carotid Intima Volume and Stenosis in *Npr2*$^{+/-}$ Mice

In a previous study we reported a strong correlation between increase in LCA intima volume and LCA (intima+media)/EEL×100% in 5 inbred mouse strains, as also seen in human atherosclerosis.[12] In the present work we found a significant correlation (R=0.6959, P<0.001) between LCA intima volume and (intima+media)/EEL×100% among 30 inbred strains of mice (Figure 4A). However, this correlation was not significant in *Npr2*$^{+/-}$ (R=0.3872) animals when compared with *Npr2*$^{+/+}$ (R=0.7928, P<0.01) littermates (Figure 4B). Furthermore, there was essentially no correlation between LCA intima volume and (intima+media)/EEL×100% in the *Npr2*$^{+/-}$ male (R=0.2362) versus *Npr2*$^{+/-}$ female (R=0.9142, P<0.05) mice (Figure 4C). These results show that even partial depletion of *Npr2* resulted in a defective carotid artery remodeling response, but only in males.

Differences in Carotid Fibrosis in *Npr2* Mice

We recently showed that pharmacologic intervention could improve carotid remodeling by inhibiting fibrosis after injury.[21] Representative images of LCA cross-sections stained with Alcian Blue show remodeling differences between male *Npr2*$^{+/-}$ versus male *Npr2*$^{+/+}$ (*Npr2* females are shown in insets in Figure 5A and 5B). The relative staining expression within the LCA intima/media (black brackets) was significantly greater in *Npr2*$^{+/-}$ versus male *Npr2*$^{+/+}$ and *Npr2*$^{+/-}$ females (Figure 5C). An increase in fibrosis in *Npr2*$^{+/-}$ males was also confirmed by PicroSirius Red staining of carotid arteries after ligation (Figure S7). Our data suggest that decreased carotid

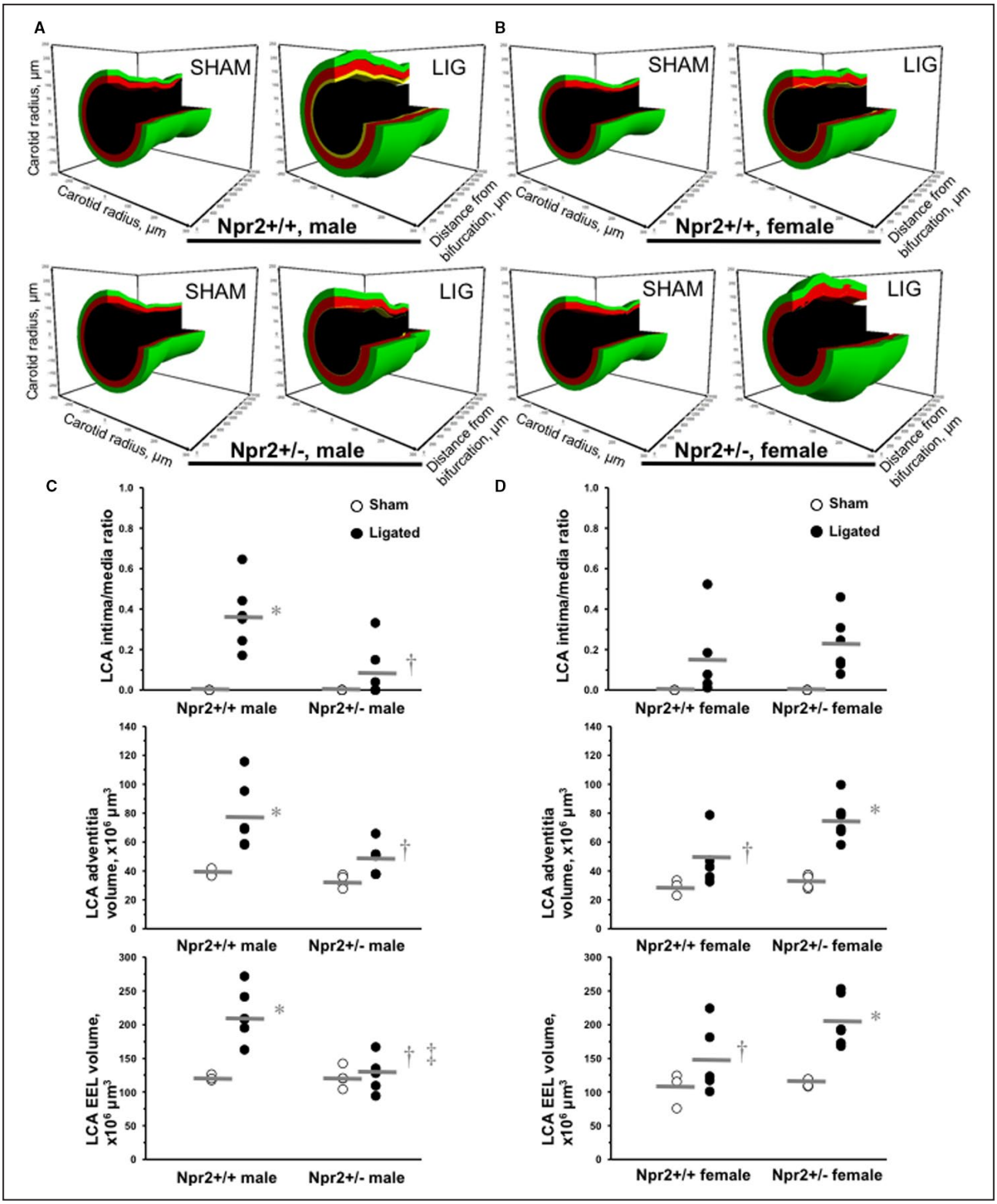

Figure 3. Flow-dependent carotid remodeling in Npr2 mice.

A, Representative 3-dimensional (3D) reconstructions of the 2-mm length from the bifurcation of the LCA after sham or ligation operation in males of *Npr2* wild-type (*Npr2*$^{+/+}$) and Npr2 heterozygous (*Npr2*$^{+/-}$) mice. **B**, Representative 3D reconstructions of LCA after sham or ligation operation in females of *Npr2*$^{+/+}$ and *Npr2*$^{+/-}$ mice. Black indicates lumen, yellow indicates intima, red indicates media, and green indicates adventitia volume. **C**, Quantifications of LCA intima/media ratio, adventitia, and EEL volume in males of *Npr2*$^{+/+}$ and *Npr2*$^{+/-}$ mice. **D**, Quantifications of LCA intima/media ratio, adventitia, and EEL volume in females of *Npr2*$^{+/+}$ and *Npr2*$^{+/-}$ mice. Open circles indicate individual sham LCAs. Black circles indicate ligated LCAs. Gray lines indicate mean values. EEL indicates external elastic lamina; and LCA, left carotid artery. *P<0.05 vs sham; †P<0.05 vs *Npr2*$^{+/+}$ males; ‡P<0.05 vs *Npr2*$^{+/-}$ females. n=3 to 6 per group.

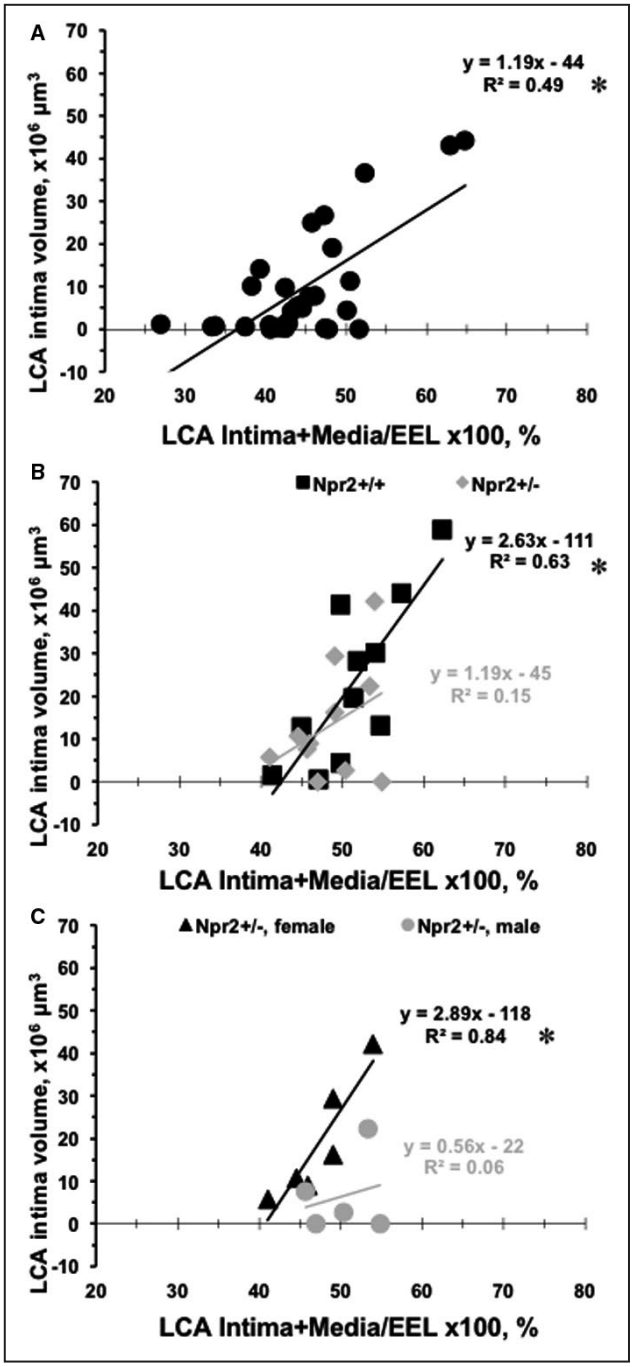

Figure 4. Relationships between carotid remodeling traits in mice.
A, Ligated LCA (intima+media)/EEL×100% on *X*-axis are plotted vs LCA intima volumes on *Y*-axis for each of 30 inbred mouse strains (black circles). **B**, Ligated LCA (intima+media)/EEL×100% on the *X*-axis are plotted vs LCA intima volumes on *Y*-axis for *Npr2* wild-type (black squares: $Npr2^{+/+}$) and Npr2 heterozygous (gray diamonds: $Npr2^{+/-}$) mice. **C**, Ligated LCA (intima+media)/EEL×100% on *X*-axis are plotted vs LCA intima volumes on the *Y*-axis for $Npr2^{+/-}$ females (black triangles) and $Npr2^{+/-}$ males (gray circles). **P*<0.001, †*P*<0.01, and ‡*P*<0.05 indicate level of significance of correlation between LCA (intima+media)/EEL×100% and LCA intima volume. EEL indicates external elastic lamina; and LCA, left carotid artery.

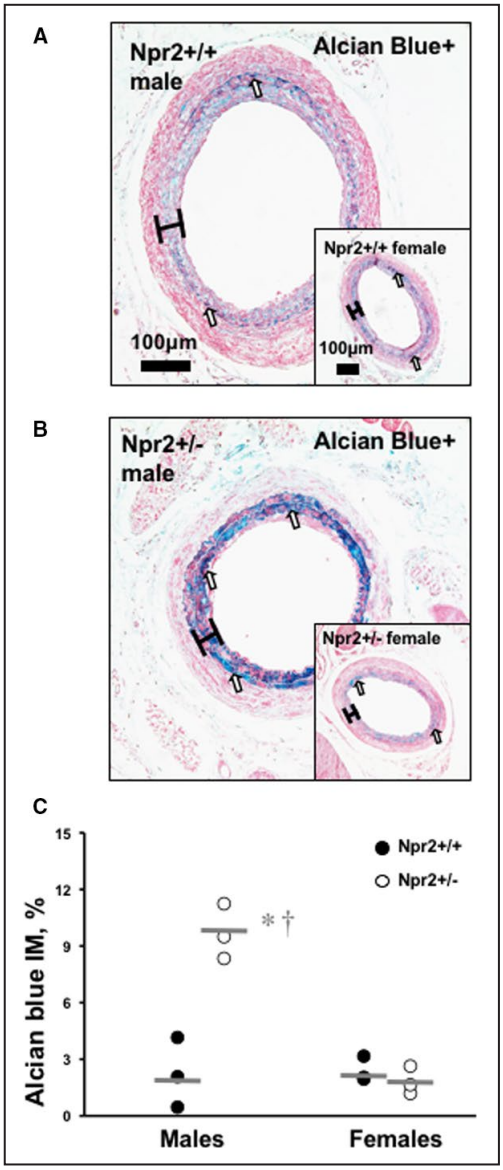

Figure 5. Differences in carotid fibrosis in Npr2 mice.
A, Representative image of PicroSirius Red–stained ligated LCA in male *Npr2* wild-type ($Npr2^{+/+}$) mouse. **B**, Representative image of Alcian Blue–stained ligated LCA in male *Npr2* heterozygous ($Npr2^{+/-}$) mouse. Insets show corresponding females. Scale bar=100 µm. Black brackets indicate intima/media area. **C**, Quantification of fibrosis (blue color) in intima/media area of the LCA (%). Black circles indicate individual $Npr2^{+/+}$ mice. Open circles indicate $Npr2^{+/-}$ mice. Gray lines indicate mean values. **P*<0.001 vs $Npr2^{+/+}$ males; †*P*<0.001 vs $Npr2^{+/-}$ females. n=3 animals per group. LCA indicates left carotid artery.

remodeling in male $Npr2^{+/-}$ mice is, in part, due to an increase in vascular fibrosis.

Decreased NPR2 Expression in Human Atherosclerotic Plaque

We identified a significant 4-fold reduction in $NPR2^{+}$ immunoreactivity within the atherosclerotic

lesions (dark bar) compared with the medial compartment (white brackets) of human carotid artery (Figure 6A). The NPR2 protein expression profile showed the same decline as a smooth muscle–specific staining (smooth muscle actin^{+}) in human lesions (Figure 6B). In contrast, macrophage-specific staining (CD68^{+}) was increased in lesions compared with media in human endarterectomy samples (Figure 6C).

DISCUSSION

The significant variation in response to vascular injury across 30 inbred mouse strains allowed us to identify 12 common SNPs associated with variation in LCA intima volume, intima/media ratio, and (intima+media)/EEL×100% traits. Our findings suggest that the gene discovered, *Npr2*, is a plausible candidate for regulation of carotid remodeling traits in response to vascular

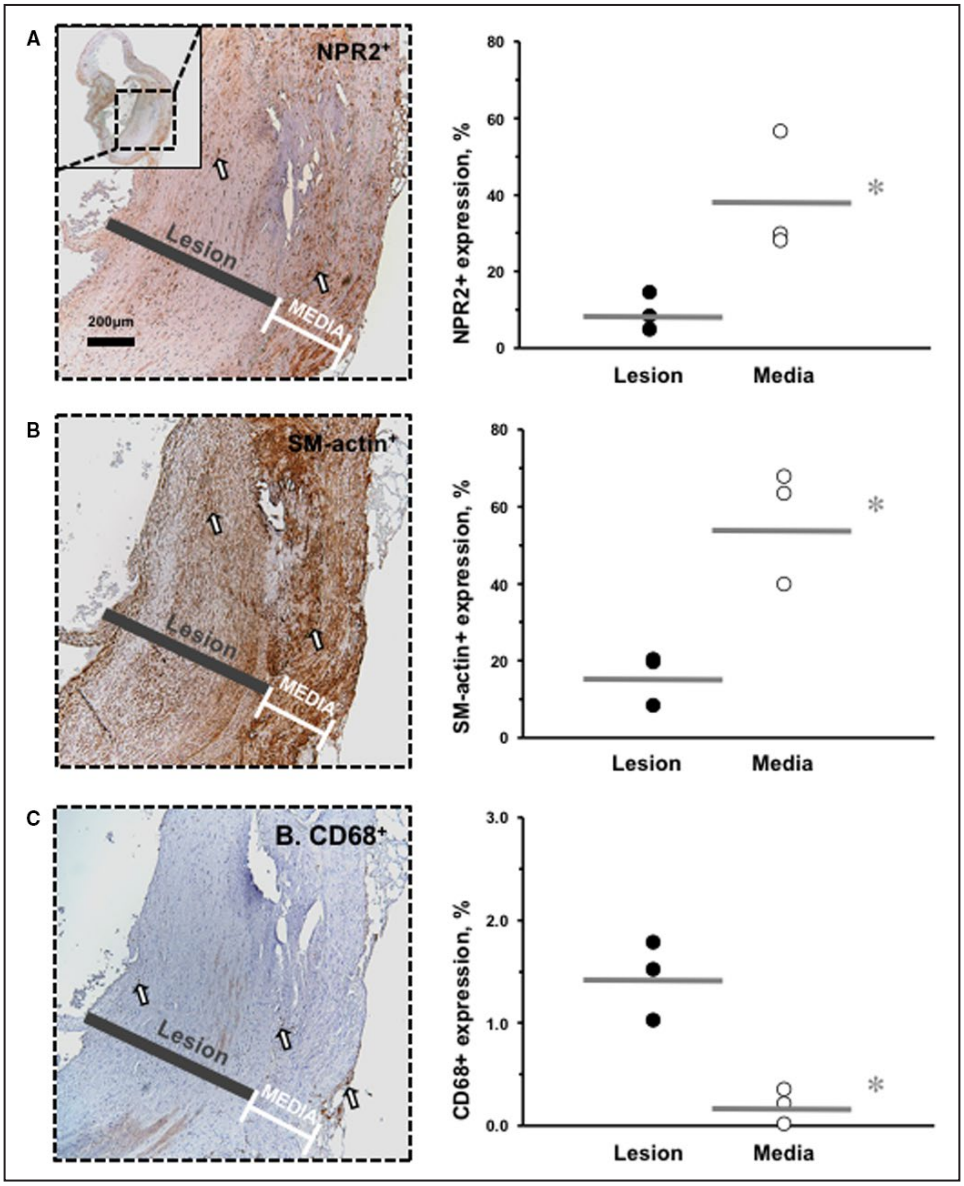

Figure 6. Decreased NPR2 expression in smooth muscle cells in human carotid lesions.
A, NPR2 immunostaining. Inset shows lower power of the carotid endarterectomy sample. **B**, α1-SM-actin immunostaining. **C**, CD68 immunostaining. Positive cells are stained brown (open arrows). Black bars indicate lesion area. White brackets indicate media area. Magnification bar=200 μm. For quantification, black circles show relative expression in the lesion of the carotid endarterectomy samples and open circles indicate media of the carotid endarterectomy samples. Gray lines indicate mean values. *P<0.05 vs media; †P<0.01 vs media. n=3 animals per group. SM indicates smooth muscle.

injury. Indeed, *Npr2*$^{+/-}$ mice had significantly less intima and EEL volumes than *Npr2*$^{+/+}$ mice. We also found greater fibrosis in carotid IMT in *Npr2*$^{+/-}$ mice, but both of these changes were significant only in *Npr2*$^{+/-}$ males.

GWA identified several QTLs on human chr7q, chr12q, and chr14q that influence carotid IMT variation in the Framingham Heart and Dominican Family studies.[5,6] A follow-up GWA mapping of the maximum common carotid IMT revealed 11 SNPs, including a proprotein convertase subtilisin/kexin type 2 in the Framingham Heart Study.[2] A similar number of SNPs were associated with carotid IMT in a cohort from China.[3] Despite advances in uncovering genetic causes of the carotid IMT, human genetics studies suffer from lack of power and technical limitations. An alternative for identifying new regulatory elements for human carotid IMT are experiments in animal models of carotid artery injury or atherosclerosis.[13,16,32,33] A primary QTL on mouse chr12, named *Cath1*, in addition to loci on chr5, chr9, and chr13, contribute to carotid atherosclerosis based on meta-analysis from 3 genetic crosses between BALB/cJ, C57BL/6J, C3H/HeJ, and SM/J inbred mouse strains on the apolipoprotein E$^{-/-}$ background.[16–18] A carotid neointima hyperplasia locus in response to injury was also detected on mouse chr12 in the genetic cross between C57BL/6H.*ApoE*$^{-/-}$ and C3H/HeJ.*ApoE*$^{-/-}$ mice, which was independent from systemic inflammation.[33] In addition to carotid atherosclerosis mouse models, we developed a mouse model of low-flow–induced carotid IMT.[11,12] We believe that this model resembles early events in vascular remodeling that are associated with progression of human carotid IMT.[34] Our group discovered 3 significant carotid intima modifier loci on mouse chr2, chr11, and chr18 in response to low blood flow in a genetic cross between C3HeB/FeJ and SJL/J mice.[13] We confirmed that the *Im2* locus on mouse chr11 contributes to carotid intima inflammation using the congenic mapping approach.[14,15] Similar to the linkage and GWA for carotid IMT in humans, our GWA results in mice reveal new candidates on chr1, chr4, and chr12 that control carotid remodeling in response to low blood flow. The comprehensive analyses of genetic regulation of the compartments of the carotid IMT allowed us to find common SNPs that associate with intima volume, intima/media ratio, and (intima+media)/EEL×100% traits. Among the candidates, *Npr2* was previously identified as a BP candidate gene on chr4 and linked to essential hypertension in humans.[30,31] Our findings further support polygenic control of carotid artery remodeling and suggest *Npr2* as a plausible candidate.

A targeted deletion of exons 3 through 7, which encode the carboxyl-terminal half of the extracellular domain and transmembrane segment of the *Npr2*, resulted in dwarfism and female sterility in *Npr2*$^{-/-}$ mice.[23] A rare genetic disorder, acromesomelic dysplasia, type Maroteaux, presents with short-limbed dwarfism after homozygous loss-of-function mutations in human *NPR2.*[35] The highest association of the *NPR2* was found with body height and fibrinogen levels in a large-scale GWA, which is relevant to earlier findings in a small genetic study in humans and after *Npr2* perturbation in mice.[23,35] *Npr2*, also known as *Npr-B*, belongs to a family of natriuretic peptide–binding proteins and represents 1 of the 5 transmembrane guanylyl cyclases found in humans.[36] A primary ligand for Npr2, C-type natriuretic peptide, can relax aortic rings, probably by binding to Npr2 and increasing cyclic guanosine monophosphate production that activates protein kinase GI, which phosphorylates target proteins.[37] A downstream target of Npr2-dependent signaling, protein kinase GI phosphorylates and activates a myosin light-chain phosphatase that increases the calcium levels necessary for cell contraction, which lowers calcium sensitivity. Activation of the Npr2/cyclic guanosine monophosphate axis in pericytes is responsible for relaxation of precapillary arterioles and capillaries.[38] Genetic studies in mice suggested that C-type natriuretic peptide and *Npr2* are candidate genes in salt-induced BP.[30] We and others showed that *Npr2* has no significant role in BP homeostasis in mice.[22,23] However, experiments in genetically manipulated mice, hypertensive rats, and in a large human GWA study showed that C-type natriuretic peptide production by endothelial cells is most important for reduction of BP.[23,39–42] Herein, we found that *Npr2* is critical for adaptation of the carotid artery in response to vascular injury. A striking difference in carotid sizes in *Npr2*$^{+/-}$ mice was also related to a significant decrease in intima volume. A moderate increase in carotid fibrosis in *Npr2*$^{+/-}$ mice resulted in a constrictive carotid phenotype in a nonfibrotic C57BL/6 background, as we recently reported.[21] These data support our idea of genetic regulation of the unique cellular and biochemical processes in carotid artery disease.[43] Our GWA findings are supported by the alteration in carotid remodeling in *Npr2*$^{+/-}$ mice and significant reduction of NPR2 expression in human atherosclerotic plaque. We believe that future clinical studies will uncover Npr2-mediated mechanisms of carotid IMT in humans.

Another significant finding in our study is that male, but not female, *Npr2*$^{+/-}$ mice exhibited constrictive carotid artery remodeling in response to low blood flow. The molecular basis for sex bias in carotid IMT development may be because of direct vasoprotective properties of estrogen on endothelial cells[44–46] by antagonizing inflammatory responses such as tumor necrosis factor-α signaling.[47] For example, in a surgical injury model, female mice had a >90% reduction in carotid intima formation relative to males, which was attenuated by ovariectomy.[48] Functional

genetic studies in rodents have identified several sexual dimorphic factors contributing to carotid remodeling that were not apparent from human data, including lower endothelial nitric oxide synthase messenger RNA levels in female aorta versus male, and less oxidized phospholipid levels in females.[49,50] Intriguingly, greater carotid pathologic remodeling in male mice mirrors sexual dimorphism in vascular injury response and disease in humans. Data from the AXA study, the Gutenberg Heart study, and an Okinawa–Nagano study revealed that men have higher carotid IMT levels relative to women.[51–53] Furthermore, several risk factors for carotid IMT display sexual dimorphism: In the Tromso study, fibrinogen levels and amount of physical activity were associated with carotid IMT in men only, whereas triglyceride levels were associated with carotid IMT in women only.[54,55] Other risk factors for carotid IMT, such as age, systolic BP, HDL, total cholesterol, body mass index, and smoking, did not correlate strongly with carotid IMT in a sex-specific manner.[55] When combined, both human and mouse carotid artery data strongly suggest a protective role for estrogen in regulating vascular intima growth responses, which contribute to progression of carotid atherosclerosis and artery occlusion.

In conclusion, we have demonstrated the power of using mouse genetic analyses to identify candidate genes, and provide novel evidence for the role of *Npr2* in the genetic regulation of vascular fibrosis associated with increased flow-dependent carotid remodeling. Future studies will explore the underlying mechanisms by which *Npr2* regulates fibrosis toward the goal of developing new clinical therapeutic approaches to treating vascular disease.

ARTICLE INFORMATION

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Supplementary Materials

Tables S1–S8
Figures S1–S7

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SUPPLEMENTAL MATERIAL

Table S1. Changes of blood flow after carotid ligation across 30 inbred mouse strains.

Mouse strains		LCA Blood flow changes to control, %	RCA Blood flow changes to control, %
129X1/SvJ,	129X1	-82 ± 4	64 ± 13
A/J,	A	-80 ± 10	83 ± 36
AKR/J,	AKR	-80 ± 2	92 ± 18
BALB/cJ,	BALB	-82 ± 6	57 ± 35
BTBR T^{+} $Itpr3^{tf}$/J,	BTBRT	-86 ± 8	87 ± 15
BUB/BnJ,	BUB	-92 ± 3	12 ± 15
C3H/HeJ,	C3H	-79 ± 0	78 ± 24
C3HeB/FeJ,	C3HEB	-82 ± 3	155 ± 15
C57BL/6J,	C57BL	-89 ± 2	80 ± 8
C57L/J,	C57L	-88 ± 5	35 ± 16
C58/J,	C58	-93 ± 2	-13 ± 8
CBA/J,	CBA	-90 ± 4	74 ± 4
CE/J,	CE	-85 ± 4	81 ± 0
DBA/2J,	DBA	-80 ± 3	59 ± 2
FVB/NJ,	FVB	-79 ± 3	64 ± 13
I/LnJ,	ILN	-93 ± 2	103 ± 19
KK/HIJ,	KK	-95 ± 0	1 ± 16
LG/J,	LG	-72 ± 4	69 ± 18
LP/J,	LP	-92 ± 1	58 ± 12
MA/MyJ,	MA	-82 ± 7	180 ± 4
NOD/LtJ,	NOD	-87 ± 5	20 ± 4
NON/LtJ,	NON	-73 ± 8	72 ± 15
NZB/B1NJ,	NZB	-88 ± 2	78 ± 28
NZW/LacJ,	NZW	-87 ± 3	53 ± 9
PL/J,	PL	-87 ± 0	46 ± 32
RIIIS/J,	RIIIS	-95 ± 1	72 ± 15
SEA/GnJ,	SEA	-93 ± 1	66 ± 3
SJL/J,	SJL	-82 ± 10	26 ± 13
SM/J,	SM	-97 ± 1	58 ± 28
SWR/J,	SWR	-90 ± 1	-2 ± 21

LCA, left carotid artery. RCA, right carotid artery.

Table S2. Significant SNPs associated with LCA intima volume in 30 inbred mouse strains.

dbSNP	Chromosome	Position, bp	p-value	Effect size
rs38940843	6	101397279	8.84E-16	11.8286
rs39087800	6	101412875	8.84E-16	11.8286
rs47480301	16	71716616	7.98E-10	11.0697
rs48569540	12	98832072	1.36E-08	11.1906
rs27831183	4	43542462	5.19E-08	9.69708
rs28320686	4	43620641	5.19E-08	9.69708
rs28320653	4	43645470	5.19E-08	9.69708
rs28320604	4	43650523	5.19E-08	9.69708
rs28320543	4	43662649	5.19E-08	9.69708
rs28320511	4	43688855	5.19E-08	9.69708
rs28311534	4	43732460	5.19E-08	9.69708
rs36294984	12	102175044	5.43E-08	9.67966
rs36281276	12	102181524	5.43E-08	9.67966
rs36677986	1	165121248	5.59E-08	9.66759
rs37370522	1	165124350	5.59E-08	9.66759
rs38372684	1	165124376	5.59E-08	9.66759
rs47497019	16	70626697	1.36E-07	10.6743
rs39750878	19	3995639	2.04E-07	10.2718
rs37877606	19	4003976	2.04E-07	10.2718
rs51910283	14	113914779	2.10E-07	10.28
rs37621976	14	114273384	2.10E-07	10.28
rs47106961	14	114918729	2.10E-07	10.28
rs46464669	14	114921628	2.10E-07	10.28
rs45841162	14	115030414	2.10E-07	10.28
rs27605802	2	171299100	3.00E-07	10.2073
rs50433974	13	60669054	3.65E-07	9.20322
rs47419227	16	72782312	3.93E-07	9.25304
rs48298153	16	72788432	3.93E-07	9.25304
rs48931505	16	72788858	3.93E-07	9.25304
rs48184804	16	72788906	3.93E-07	9.25304
rs47318646	16	72789323	3.93E-07	9.25304
rs37071291	6	100325877	4.59E-07	10.5823

rs36697249	1	165125538	4.83E-07	9.20222
rs36283974	6	101145567	1.40E-06	10.5364
rs37705272	6	101418487	1.43E-06	10.3656
rs4203146	16	73624179	1.50E-06	8.80081
rs4203158	16	73626715	1.50E-06	8.80081
rs4203159	16	73627098	1.50E-06	8.80081
rs49791987	16	73634287	1.50E-06	8.80081
rs4203186	16	73637376	1.50E-06	8.80081
rs4203509	16	73865267	1.50E-06	8.80081
rs4203550	16	73885132	1.50E-06	8.80081
rs36371337	6	37196299	2.10E-06	10.6436
rs37712916	6	37196939	2.10E-06	10.6436
rs37118187	6	37199656	2.10E-06	10.6436
rs38226710	6	37215497	2.10E-06	10.6436
rs37864322	6	37233039	2.10E-06	10.6436
rs36742390	6	37236202	2.10E-06	10.6436
rs37223003	6	37243700	2.10E-06	10.6436
rs36251037	6	37244001	2.10E-06	10.6436
rs4203316	16	73769593	2.16E-06	8.60563
rs49296290	1	164345397	2.50E-06	9.5115
rs27916937	2	48877169	2.92E-06	9.61417
rs27918756	2	49953267	2.92E-06	9.61417
rs27918720	2	49959001	2.92E-06	9.61417
rs27918614	2	50000652	2.92E-06	9.61417
rs27918554	2	50032628	2.92E-06	9.61417
rs27918534	2	50044326	2.92E-06	9.61417
rs49704766	2	50285472	2.92E-06	9.61417
rs28295363	2	50858835	2.92E-06	9.61417
rs28295360	2	50859030	2.92E-06	9.61417
rs27930813	2	50956084	2.92E-06	9.61417
rs27602702	2	170907558	2.92E-06	9.61417
rs47868731	14	115246865	3.12E-06	9.67611
rs49720353	14	115417489	3.12E-06	9.67611
rs46884614	14	115579774	3.12E-06	9.67611
rs51463268	14	115580256	3.12E-06	9.67611
rs30604832	14	70561864	3.29E-06	9.2204

rs50052151	17	31594946	3.30E-06	9.64921
rs49741305	17	31597321	3.30E-06	9.64921
rs50385858	18	54841501	3.33E-06	9.61579
rs37738336	18	78267254	3.33E-06	9.61579
rs37234714	18	78267687	3.33E-06	9.61579
rs37792462	18	78277154	3.33E-06	9.61579
rs38103448	18	78466732	3.33E-06	9.61579
rs36981969	18	78471727	3.33E-06	9.61579
rs36387979	18	78471745	3.33E-06	9.61579
rs38025680	18	78494820	3.33E-06	9.61579
rs38606622	18	78558805	3.33E-06	9.61579
rs38648125	18	79112446	3.33E-06	9.61579
rs37191882	18	79120190	3.33E-06	9.61579
rs36853173	18	79126472	3.33E-06	9.61579
rs47705897	1	167745338	3.57E-06	8.94812
rs48039498	1	167745486	3.57E-06	8.94812
rs47739937	1	167745525	3.57E-06	8.94812

Table S3. Significant SNPs associated with LCA media volume in 30 inbred mouse strains.

dbSNP	Chromosome	Position, bp	p-value	Effect size
rs4167895	16	30058454	3.73E-06	-9.53356

Table S4. Significant SNPs associated with LCA adventitia volume in 30 inbred mouse strains.

dbSNP	Chromosome	Position, bp	p-value	Effect size
rs47073871	13	20636267	6.61E-08	12.1977
rs26883269	11	36643969	1.35E-07	11.8225
rs26883268	11	36644267	1.35E-07	11.8225
rs6223990	11	36649077	1.35E-07	11.8225
rs26883254	11	36657106	1.35E-07	11.8225
rs26883252	11	36657385	1.35E-07	11.8225
rs26883248	11	36658108	1.35E-07	11.8225
rs26883247	11	36658209	1.35E-07	11.8225
rs26883238	11	36660335	1.35E-07	11.8225
rs26883211	11	36666657	1.35E-07	11.8225
rs46269877	11	36667397	1.35E-07	11.8225
rs50176409	11	36667580	1.35E-07	11.8225
rs26883183	11	36676089	1.35E-07	11.8225
rs26933638	11	36682571	1.35E-07	11.8225
rs28212174	11	39241249	1.35E-07	11.8225
rs36410510	19	11159100	1.43E-07	12.2065
rs38678405	13	19913279	1.93E-07	11.8037
rs29607886	13	21048396	2.82E-07	11.642
rs36302909	13	38317257	3.09E-07	11.8565
rs29600146	13	20855501	3.43E-07	12.0104
rs31653947	15	25783771	4.07E-07	13.8686
rs32023348	15	25789703	4.07E-07	13.8686
rs36719773	15	25792839	4.07E-07	13.8686
rs31876896	15	25793549	4.07E-07	13.8686
rs36896622	13	19623895	4.81E-07	11.5772
rs36608337	13	19631552	4.81E-07	11.5772
rs36444549	13	19633288	4.81E-07	11.5772
rs38251579	13	19691192	4.81E-07	11.5772
rs36733180	13	19691255	4.81E-07	11.5772
rs47114836	13	19722060	4.81E-07	11.5772
rs36735954	13	19765425	4.81E-07	11.5772
rs37428520	13	19767050	4.81E-07	11.5772

rs38026141	13	19820179	4.81E-07	11.5772
rs36661788	13	19821058	4.81E-07	11.5772
rs38303318	13	19821229	4.81E-07	11.5772
rs36594603	13	19822595	4.81E-07	11.5772
rs38318713	13	19823039	4.81E-07	11.5772
rs36391344	13	19902374	4.81E-07	11.5772
rs37258331	13	19902772	4.81E-07	11.5772
rs37914692	13	19913414	4.81E-07	11.5772
rs48502495	13	19929924	4.81E-07	11.5772
rs37799072	13	19953624	4.81E-07	11.5772
rs36781926	13	19974390	4.81E-07	11.5772
rs47829231	13	20013348	4.81E-07	11.5772
rs49358850	13	20013404	4.81E-07	11.5772
rs36640438	13	20020136	4.81E-07	11.5772
rs36487463	13	20024700	4.81E-07	11.5772
rs6297328	13	20068362	4.81E-07	11.5772
rs6299142	13	20068687	4.81E-07	11.5772
rs36690570	13	20076501	4.81E-07	11.5772
rs36378954	13	20111321	4.81E-07	11.5772
rs6295123	13	20119946	4.81E-07	11.5772
rs37427421	13	20154538	4.81E-07	11.5772
rs37056179	13	20171962	4.81E-07	11.5772
rs38132771	13	20175773	4.81E-07	11.5772
rs36471029	13	20179306	4.81E-07	11.5772
rs37915568	13	20186083	4.81E-07	11.5772
rs6344576	13	20188891	4.81E-07	11.5772
rs6248419	13	20189181	4.81E-07	11.5772
rs36600957	13	20205439	4.81E-07	11.5772
rs46393433	13	20254709	4.81E-07	11.5772
rs45982807	13	20283531	4.81E-07	11.5772
rs47928896	13	20301917	4.81E-07	11.5772
rs51047263	13	20312500	4.81E-07	11.5772
rs6203049	13	20315428	4.81E-07	11.5772
rs52320861	13	20322495	4.81E-07	11.5772
rs47549236	13	20365143	4.81E-07	11.5772
rs51484684	13	20371046	4.81E-07	11.5772

rs48961319	13	20374137	4.81E-07	11.5772
rs45651638	13	20379366	4.81E-07	11.5772
rs46580187	13	20382923	4.81E-07	11.5772
rs47760603	13	20414115	4.81E-07	11.5772
rs47538017	13	20420858	4.81E-07	11.5772
rs48892010	13	20421811	4.81E-07	11.5772
rs48148404	13	20469092	4.81E-07	11.5772
rs48951151	13	20471221	4.81E-07	11.5772
rs49018822	13	20481493	4.81E-07	11.5772
rs49995885	13	20511487	4.81E-07	11.5772
rs48592377	13	20539128	4.81E-07	11.5772
rs48864424	13	20552932	4.81E-07	11.5772
rs48292864	13	20569654	4.81E-07	11.5772
rs49769390	13	20571147	4.81E-07	11.5772
rs6372032	13	20572873	4.81E-07	11.5772
rs51129769	13	20575619	4.81E-07	11.5772
rs47155818	13	20597561	4.81E-07	11.5772
rs49531202	13	20608709	4.81E-07	11.5772
rs46266953	13	20684736	4.81E-07	11.5772
rs46868266	13	20685797	4.81E-07	11.5772
rs50160653	13	20719255	4.81E-07	11.5772
rs47901231	13	20744950	4.81E-07	11.5772
rs33839559	13	20772091	4.81E-07	11.5772
rs33840365	13	20772735	4.81E-07	11.5772
rs33837937	13	20788119	4.81E-07	11.5772
rs51945080	13	20812645	4.81E-07	11.5772
rs46188139	13	20812888	4.81E-07	11.5772
rs29594544	13	20859622	4.81E-07	11.5772
rs29594550	13	20859808	4.81E-07	11.5772
rs29597333	13	20882992	4.81E-07	11.5772
rs29598246	13	20883087	4.81E-07	11.5772
rs29593003	13	20894217	4.81E-07	11.5772
rs29593889	13	20916452	4.81E-07	11.5772
rs29604183	13	20991627	4.81E-07	11.5772
rs29598075	13	20992544	4.81E-07	11.5772
rs29606454	13	21000504	4.81E-07	11.5772

rs29606460	13	21000754	4.81E-07	11.5772
rs29607083	13	21048311	4.81E-07	11.5772
rs29604312	13	21073825	4.81E-07	11.5772
rs29607857	13	21132420	4.81E-07	11.5772
rs29607860	13	21132455	4.81E-07	11.5772
rs29609872	13	21141111	4.81E-07	11.5772
rs29611566	13	21142121	4.81E-07	11.5772
rs29610464	13	21164813	4.81E-07	11.5772
rs6263463	13	21171748	4.81E-07	11.5772
rs29638163	13	21223147	4.81E-07	11.5772
rs29639114	13	21249985	4.81E-07	11.5772
rs49949392	13	21261961	4.81E-07	11.5772
rs29647362	13	21281847	4.81E-07	11.5772
rs29641986	13	21292978	4.81E-07	11.5772
rs29643897	13	21295276	4.81E-07	11.5772
rs29646900	13	21298273	4.81E-07	11.5772
rs29644942	13	21337054	4.81E-07	11.5772
rs29645930	13	21357606	4.81E-07	11.5772
rs29645533	13	21371512	4.81E-07	11.5772
rs49062226	13	21372890	4.81E-07	11.5772
rs29644278	13	21394529	4.81E-07	11.5772
rs29648727	13	21430577	4.81E-07	11.5772
rs29652368	13	21434110	4.81E-07	11.5772
rs29653437	13	21465037	4.81E-07	11.5772
rs29654897	13	21507746	4.81E-07	11.5772
rs29649704	13	21522488	4.81E-07	11.5772
rs29650319	13	21547080	4.81E-07	11.5772
rs48094376	13	21558999	4.81E-07	11.5772
rs46647627	13	21559343	4.81E-07	11.5772
rs6355688	13	21576688	4.81E-07	11.5772
rs6353554	13	21609738	4.81E-07	11.5772
rs6354633	13	21609929	4.81E-07	11.5772
rs29651488	13	21627510	4.81E-07	11.5772
rs29657594	13	21658122	4.81E-07	11.5772
rs29658436	13	21659208	4.81E-07	11.5772
rs29655552	13	21676021	4.81E-07	11.5772

rs29652356	13	21700527	4.81E-07	11.5772
rs29658054	13	21752519	4.81E-07	11.5772
rs29652814	13	21758217	4.81E-07	11.5772
rs47150906	13	52211251	4.81E-07	11.5772
rs46309028	13	52211325	4.81E-07	11.5772
rs48741080	13	20962352	7.16E-07	11.7107
rs47509073	1	77255403	7.61E-07	11.7479
rs32950486	1	77609490	7.61E-07	11.7479
rs36872577	1	78067450	7.61E-07	11.7479
rs6407549	1	78079879	7.61E-07	11.7479
rs48353307	12	97613323	9.44E-07	11.641
rs52231845	13	18838734	1.25E-06	12.1417
rs4207614	16	77738835	1.26E-06	13.9067
rs4207615	16	77738908	1.26E-06	13.9067
rs48357981	16	77739452	1.26E-06	13.9067
rs4207617	16	77744195	1.26E-06	13.9067
rs48226387	16	77749862	1.26E-06	13.9067
rs4207656	16	77773830	1.26E-06	13.9067
rs4207659	16	77774195	1.26E-06	13.9067
rs46962439	16	77795529	1.26E-06	13.9067
rs4207710	16	77821575	1.26E-06	13.9067
rs4207711	16	77823818	1.26E-06	13.9067
rs4207712	16	77824526	1.26E-06	13.9067
rs48139016	15	48841397	1.53E-06	11.976
rs46494410	15	48856141	1.53E-06	11.976
rs47208714	15	49205685	1.53E-06	11.976
rs37217592	1	94081116	3.28E-06	11.5744
rs36408043	1	94147963	3.28E-06	11.5744
rs37952899	1	94169057	3.28E-06	11.5744
rs31116994	14	68371424	3.74E-06	11.5098

Table S5. Significant SNPs associated with LCA EEL volume in 30 inbred mouse strains.

dbSNP	Chromosome	Position, bp	p-value	Effect size
rs32595130	9	44253578	5.08E-07	27.908
rs32598526	9	44276639	5.08E-07	27.908
rs49698155	15	27049944	1.66E-06	27.0288
rs33417562	17	12417424	2.24E-06	-27.0414
rs31653947	15	25783771	3.12E-06	29.1715
rs32023348	15	25789703	3.12E-06	29.1715
rs36719773	15	25792839	3.12E-06	29.1715
rs31876896	15	25793549	3.12E-06	29.1715

Table S6. Significant SNPs associated with LCA intima-to-media ratio in 30 inbred mouse strains.

dbSNP	Chromosome	Position, bp	p-value	Effect size
rs27674148	4	7102117	2.80E-13	0.211489
rs27737825	4	29648251	2.80E-13	0.211489
rs36677986	1	165121248	1.01E-12	0.204791
rs37370522	1	165124350	1.01E-12	0.204791
rs38372684	1	165124376	1.01E-12	0.204791
rs36294984	12	102175044	1.02E-12	0.204791
rs36281276	12	102181524	1.02E-12	0.204791
rs27831183	4	43542462	1.04E-12	0.20448
rs28320686	4	43620641	1.04E-12	0.20448
rs28320653	4	43645470	1.04E-12	0.20448
rs28320604	4	43650523	1.04E-12	0.20448
rs28320543	4	43662649	1.04E-12	0.20448
rs28320511	4	43688855	1.04E-12	0.20448
rs28311534	4	43732460	1.04E-12	0.20448
rs38940843	6	101397279	8.49E-11	0.206547
rs39087800	6	101412875	8.49E-11	0.206547
rs50242724	3	34405923	1.06E-10	0.200376
rs47993102	15	7033810	9.69E-10	0.19749
rs27134887	2	16236183	1.63E-09	0.193885
rs27605802	2	171299100	2.21E-09	0.206106
rs36739925	9	39493014	2.25E-09	0.194116
rs32998299	7	56915547	4.48E-09	0.199285
rs46101910	7	57451000	4.48E-09	0.199285
rs38361786	7	57470793	4.48E-09	0.199285
rs27648232	2	179835451	4.53E-09	0.199145
rs27678709	2	179869730	4.53E-09	0.199145
rs36484548	14	116035561	6.04E-09	0.199359
rs37878104	14	116035938	6.04E-09	0.199359
rs36578717	14	116037357	6.04E-09	0.199359
rs50464390	14	116263623	6.04E-09	0.199359
rs36670143	14	116349890	6.04E-09	0.199359
rs38987302	14	116417450	6.04E-09	0.199359

rs47765000	14	117018624	6.04E-09	0.199359
rs48764245	16	30572961	6.06E-09	0.200082
rs48527511	16	30588279	6.06E-09	0.200082
rs50991760	16	30619586	6.06E-09	0.200082
rs37125075	18	68612691	6.30E-09	0.198834
rs36597862	18	68685395	6.30E-09	0.198834
rs27689045	4	7044109	6.32E-09	0.198671
rs27673999	4	7137905	6.32E-09	0.198671
rs27673998	4	7137926	6.32E-09	0.198671
rs27657948	4	7170558	6.32E-09	0.198671
rs27709097	4	7211496	6.32E-09	0.198671
rs27709075	4	7213943	6.32E-09	0.198671
rs27691238	4	7232617	6.32E-09	0.198671
rs27687701	4	7583955	6.32E-09	0.198671
rs27737810	4	29656779	6.32E-09	0.198671
rs27737803	4	29677730	6.32E-09	0.198671
rs27723164	4	29794635	6.32E-09	0.198671
rs6237544	4	29795573	6.32E-09	0.198671
rs27748033	4	29997199	6.32E-09	0.198671
rs27786591	4	30541140	6.32E-09	0.198671
rs27786545	4	30566381	6.32E-09	0.198671
rs49665559	4	30804028	6.32E-09	0.198671
rs27741284	4	31055741	6.32E-09	0.198671
rs27612140	4	139882104	6.32E-09	0.198671
rs27612099	4	139892826	6.32E-09	0.198671
rs27552432	4	142064805	6.32E-09	0.198671
rs27552330	4	142086314	6.32E-09	0.198671
rs36240801	6	100082220	6.55E-09	0.199186
rs27691242	4	7229815	6.70E-09	0.201547
rs47419227	16	72782312	9.72E-09	0.183091
rs48298153	16	72788432	9.72E-09	0.183091
rs48931505	16	72788858	9.72E-09	0.183091
rs48184804	16	72788906	9.72E-09	0.183091
rs47318646	16	72789323	9.72E-09	0.183091
rs27044336	11	118808786	8.12E-08	0.183893
rs26996028	11	120310104	8.12E-08	0.183893

rs31460283	14	75499722	8.35E-08	0.183679
rs27657799	4	7196977	8.37E-08	0.183807
rs32921099	12	99458647	1.31E-07	0.185488
rs27567843	4	139409395	1.71E-07	0.187519
rs33689626	1	155345800	2.21E-07	0.182525
rs51833298	12	116994028	2.60E-07	0.180779
rs45951608	12	116994377	2.60E-07	0.180779
rs6218946	11	119001637	2.82E-07	0.180494
rs27029509	11	119843831	2.82E-07	0.180494
rs50185419	16	68925430	2.95E-07	0.180757
rs47397360	17	14392836	3.48E-07	0.181558
rs31317930	14	73703911	3.91E-07	0.186536
rs37071291	6	100325877	3.95E-07	0.198601
rs46912210	3	32213292	4.22E-07	0.188115
rs50599709	3	32214276	4.22E-07	0.188115
rs51153728	3	32214591	4.22E-07	0.188115
rs48838546	10	118122159	4.86E-07	0.177113
rs36996707	9	65937308	5.07E-07	0.196914
rs27079841	2	4508832	5.62E-07	0.179491
rs27108869	2	10254363	5.62E-07	0.179491
rs27163089	2	14471057	5.62E-07	0.179491
rs27846958	4	45447167	6.11E-07	0.16913
rs51350682	17	48110423	6.68E-07	0.178579
rs27916937	2	48877169	7.66E-07	0.189528
rs27918756	2	49953267	7.66E-07	0.189528
rs27918720	2	49959001	7.66E-07	0.189528
rs27918614	2	50000652	7.66E-07	0.189528
rs27918554	2	50032628	7.66E-07	0.189528
rs27918534	2	50044326	7.66E-07	0.189528
rs49704766	2	50285472	7.66E-07	0.189528
rs28295363	2	50858835	7.66E-07	0.189528
rs28295360	2	50859030	7.66E-07	0.189528
rs27930813	2	50956084	7.66E-07	0.189528
rs27602702	2	170907558	7.66E-07	0.189528
rs51654688	14	74098899	8.28E-07	0.168017
rs36697249	1	165125538	8.34E-07	0.168486

rs47868731	14	115246865	8.56E-07	0.189731
rs49720353	14	115417489	8.56E-07	0.189731
rs46884614	14	115579774	8.56E-07	0.189731
rs51463268	14	115580256	8.56E-07	0.189731
rs50433974	13	60669054	9.01E-07	0.165023
rs50052151	17	31594946	9.13E-07	0.189667
rs49741305	17	31597321	9.13E-07	0.189667
rs50385858	18	54841501	9.48E-07	0.188616
rs37738336	18	78267254	9.48E-07	0.188616
rs37234714	18	78267687	9.48E-07	0.188616
rs37792462	18	78277154	9.48E-07	0.188616
rs38103448	18	78466732	9.48E-07	0.188616
rs36981969	18	78471727	9.48E-07	0.188616
rs36387979	18	78471745	9.48E-07	0.188616
rs38025680	18	78494820	9.48E-07	0.188616
rs38606622	18	78558805	9.48E-07	0.188616
rs38648125	18	79112446	9.48E-07	0.188616
rs37191882	18	79120190	9.48E-07	0.188616
rs36853173	18	79126472	9.48E-07	0.188616
rs6227786	9	65041134	1.10E-06	0.193507
rs50974505	14	113243033	1.48E-06	0.193465
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rs46464669	14	114921628	1.93E-06	0.187818
rs45841162	14	115030414	1.93E-06	0.187818
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rs37215995	5	131251642	2.83E-06	0.187513
rs36989849	5	131371412	2.83E-06	0.187513
rs37498312	5	131371431	2.83E-06	0.187513
rs37355302	5	131375527	2.83E-06	0.187513
rs37253900	5	131375972	2.83E-06	0.187513
rs37100728	5	131400484	2.83E-06	0.187513
rs36391382	5	131400832	2.83E-06	0.187513
rs38388261	5	131403419	2.83E-06	0.187513
rs31201166	14	72163050	2.84E-06	0.1891
rs47865024	19	3125547	2.87E-06	0.186874
rs37406791	19	3290669	2.87E-06	0.186874
rs36463751	19	3430247	2.87E-06	0.186874
rs36739976	19	3613434	2.87E-06	0.186874
rs37552721	19	5168078	2.87E-06	0.186874
rs37361176	19	5191219	2.87E-06	0.186874
rs36396226	19	5193282	2.87E-06	0.186874
rs37209662	19	5228289	2.87E-06	0.186874
rs38152662	19	5343175	2.87E-06	0.186874
rs36979098	19	5433187	2.87E-06	0.186874
rs37399532	19	5515642	2.87E-06	0.186874
rs36464166	19	5619157	2.87E-06	0.186874
rs38870454	1	9690292	2.90E-06	0.187437
rs30737389	1	120556824	2.90E-06	0.187437
rs45920082	1	121034640	2.90E-06	0.187437
rs32868148	9	65094739	3.01E-06	0.188935
rs50469542	9	66416150	3.01E-06	0.188935
rs27574360	4	133884437	3.24E-06	0.186498
rs27578237	4	134271709	3.24E-06	0.186498
rs27578194	4	134283929	3.24E-06	0.186498
rs27578147	4	134306671	3.24E-06	0.186498
rs27578114	4	134315926	3.24E-06	0.186498
rs27578111	4	134316358	3.24E-06	0.186498

rs27578022	4	134336464	3.24E-06	0.186498
rs31846156	15	87292777	3.37E-06	0.173925
rs32108716	12	66694958	3.40E-06	0.189138
rs51955425	16	72788939	3.53E-06	0.183817
rs4203146	16	73624179	3.57E-06	0.160604
rs4203158	16	73626715	3.57E-06	0.160604
rs4203159	16	73627098	3.57E-06	0.160604
rs49791987	16	73634287	3.57E-06	0.160604
rs4203186	16	73637376	3.57E-06	0.160604
rs4203509	16	73865267	3.57E-06	0.160604
rs4203550	16	73885132	3.57E-06	0.160604
rs51994823	17	33766971	3.65E-06	0.169664
rs49629788	17	33767492	3.65E-06	0.169664
rs51346762	17	33791241	3.65E-06	0.169664
rs46439310	17	34046644	3.65E-06	0.169664
rs26828028	11	31235263	3.74E-06	0.167762
rs52649558	12	42642305	3.75E-06	0.183467
rs32122316	12	66822676	3.86E-06	0.194331

Table S7. Significant SNPs associated with LCA intima+media/EELx100% in 30 inbred mouse strains.

dbSNP	Chromosome	Position, bp	p-value	Effect size
rs36294984	12	102175044	1.64E-07	5.35241
rs36281276	12	102181524	1.64E-07	5.35241
rs36677986	1	165121248	1.68E-07	5.35456
rs37370522	1	165124350	1.68E-07	5.35456
rs38372684	1	165124376	1.68E-07	5.35456
rs27831183	4	43542462	1.72E-07	5.35142
rs28320686	4	43620641	1.72E-07	5.35142
rs28320653	4	43645470	1.72E-07	5.35142
rs28320604	4	43650523	1.72E-07	5.35142
rs28320543	4	43662649	1.72E-07	5.35142
rs28320511	4	43688855	1.72E-07	5.35142
rs28311534	4	43732460	1.72E-07	5.35142
rs49300701	12	70834268	4.29E-07	5.28592
rs32825856	12	98293756	4.29E-07	5.28592
rs32826743	12	98311790	4.29E-07	5.28592
rs32831405	12	98324424	4.29E-07	5.28592
rs32832502	12	98431791	4.29E-07	5.28592
rs32833315	12	98431830	4.29E-07	5.28592
rs32835668	12	98440111	4.29E-07	5.28592
rs27077445	11	92536007	6.75E-07	5.31958
rs47184414	13	63671652	1.56E-06	5.28121
rs50150006	13	63672826	1.56E-06	5.28121
rs51457735	13	63843692	1.56E-06	5.28121
rs50629131	13	64083663	1.56E-06	5.28121
rs31846156	15	87292777	1.62E-06	5.2561
rs27077330	11	92579730	3.95E-06	4.97734
rs27077326	11	92580059	3.95E-06	4.97734
rs27077313	11	92580950	3.95E-06	4.97734
rs27077297	11	92594015	3.95E-06	4.97734
rs27062266	11	92606779	3.95E-06	4.97734
rs4140244	11	92609707	3.95E-06	4.97734
rs27062241	11	92609733	3.95E-06	4.97734

Table S8. Blood flow profiles in the left carotid arteries across the groups.

Groups	Parameters	Mean Velocity mm/s	End Diastolic Velocity mm/s	Peak Systolic velocity mm/s	VTI mm	Mean Gradient mmHg	Pulsatility index
Npr2+/+ n=5	**Sham**	303 ± 63	135 ± 24	521 ± 107	64 ± 11	0.430 ± 0.181	1.27 ± 0.05
	Ligated	38 ± 6 *	5 ± 1 *	74 ± 13 *	6 ± 0 *	0.006 ± 0.002 *	1.79 ± 0.11 *
Npr2+/- n=4	**Sham**	233 ± 28	119 ± 15	405 ± 50	51 ± 3	0.227 ± 0.057	1.21 ± 0.11
	Ligated	42 ± 15 *	12 ± 4 *	87 ± 29 *	6 ± 1 *	0.010 ± 0.006	1.78 ± 0.14 *

n, Number of mice. *, $p<0.05$ vs. proper sham group.

Supplemental Figure Legends:

Figure S1. Variation in carotid remodeling in ten inbred mouse strains. Representative 3-dimensional reconstructions of the 2mm-length from the bifurcation of the left carotid artery after sham (SHAM) or ligation (LIG) operation in males of 129X1/SvJ (129X1), A/J (A), AKR/J (AKR), BALB/cJ (BALB), BTBR T+ Itpr3tf/J (BTBRT), BUB/BnJ (BUB), C3H/HeJ (C3H), C3HeB/FeJ (C3HEB), C57BL/6J (C57BL), C57L/J (C57L) mice. Black color shows lumen, yellow – intima, red – media, green – adventitia volume.

Figure S2. Variation in carotid remodeling in ten inbred mouse strains. Representative 3-dimensional reconstructions of the 2mm-length from the bifurcation of the left carotid artery after sham (SHAM) or ligation (LIG) operation in males of C58/J (C58), CBA/J (CBA), CE/J (CE), DBA/2J (DBA), FVB/NJ (FVB), I/LnJ (ILN), KK/HIJ (KK), LG/J (LG), LP/J (LP), MA/MyJ (MA) mice. Black color shows lumen, yellow – intima, red – media, green – adventitia volume.

Figure S3. Variation in carotid remodeling in ten inbred mouse strains. Representative 3-dimensional reconstructions of the 2mm-length from the bifurcation of the left carotid artery after sham (SHAM) or ligation (LIG) operation in males of NOD/LtJ (NOD), NON/LtJ (NON), NZB/B1NJ (NZB), NZW/LacJ (NZW), PL/J (PL), RIIIS/J (RIIIS), SEA/GnJ (SEA), SJL/J (SJL), SM/J (SM), SWR/J (SWR) mice. Black color shows lumen, yellow – intima, red – media, green – adventitia volume.

Figure S4. Genome-wide association (GWA) of the left carotid artery (LCA) media trait in 30 mouse strains. A. GWA of LCA media volume, $x10^{-6}\,\mu m^3$. Open circles are controls. Black circles are ligated mice. Values are mean±SEM; *, $p<0.05$ vs. control or other mouse strains. n=4-6 per group. **B.** GWA of LCA media volume. Each circle represents a SNP. Mouse

chromosomes are on X-axis. Gray line shows a threshold of significance.

Figure S5. Genome-wide association (GWA) of the left carotid artery (LCA) external elastic lamina (EEL) trait in 30 mouse strains. A. GWA of LCA EEL volume, $x10^{-6}$ μm^3. Open circles are controls. Black circles are ligated mice. Values are mean±SEM; *, $p<0.05$ vs. control or other mouse strains. n=4-6 per group. **B.** GWA of LCA EEL volume. Each circle represents a SNP. Mouse chromosomes are on X-axis. Gray line shows a threshold of significance.

Figure S6. Genome-wide association (GWA) of the left carotid artery (LCA) adventitia trait in 30 mouse strains. A. GWA of LCA adventitia volume, $x10^{-6}$ μm^3. Open circles are controls. Black circles are ligated mice. Values are mean±SEM; *, $p<0.05$ vs. control or other mouse strains. n=4-6 per group. **B.** GWA of LCA adventitia volume. Each circle represents a SNP. Mouse chromosomes are on X-axis. Gray line shows a threshold of significance.

Figure S7. Increases in PicroSirius Red staining in male *Npr2*$^{+/-}$ mice. A. A representative image of PicroSirius Red-stained ligated left carotid artery (LCA) in male *Npr2* wild type (*Npr2*$^{+/+}$) mouse. **B.** A representative image of PicroSirius Red-stained ligated LCA in male *Npr2* heterozygous (*Npr2*$^{+/-}$) mouse. Insets are corresponding females. Scale bar is 100μm. Black brackets indicate intima-media area. **C.** A quantification of fibrosis (red color) in intima- media area of the LCA, %. Individual *Npr2*$^{+/+}$ mice are shown as black circles. Open circles indicate *Npr2*$^{+/-}$ mice. Gray lines are mean values; *, $p<0.05$ vs. *Npr2*$^{+/+}$ males; †, $p<0.05$ vs. *Npr2*$^{+/-}$ females. n=3 animals per group.

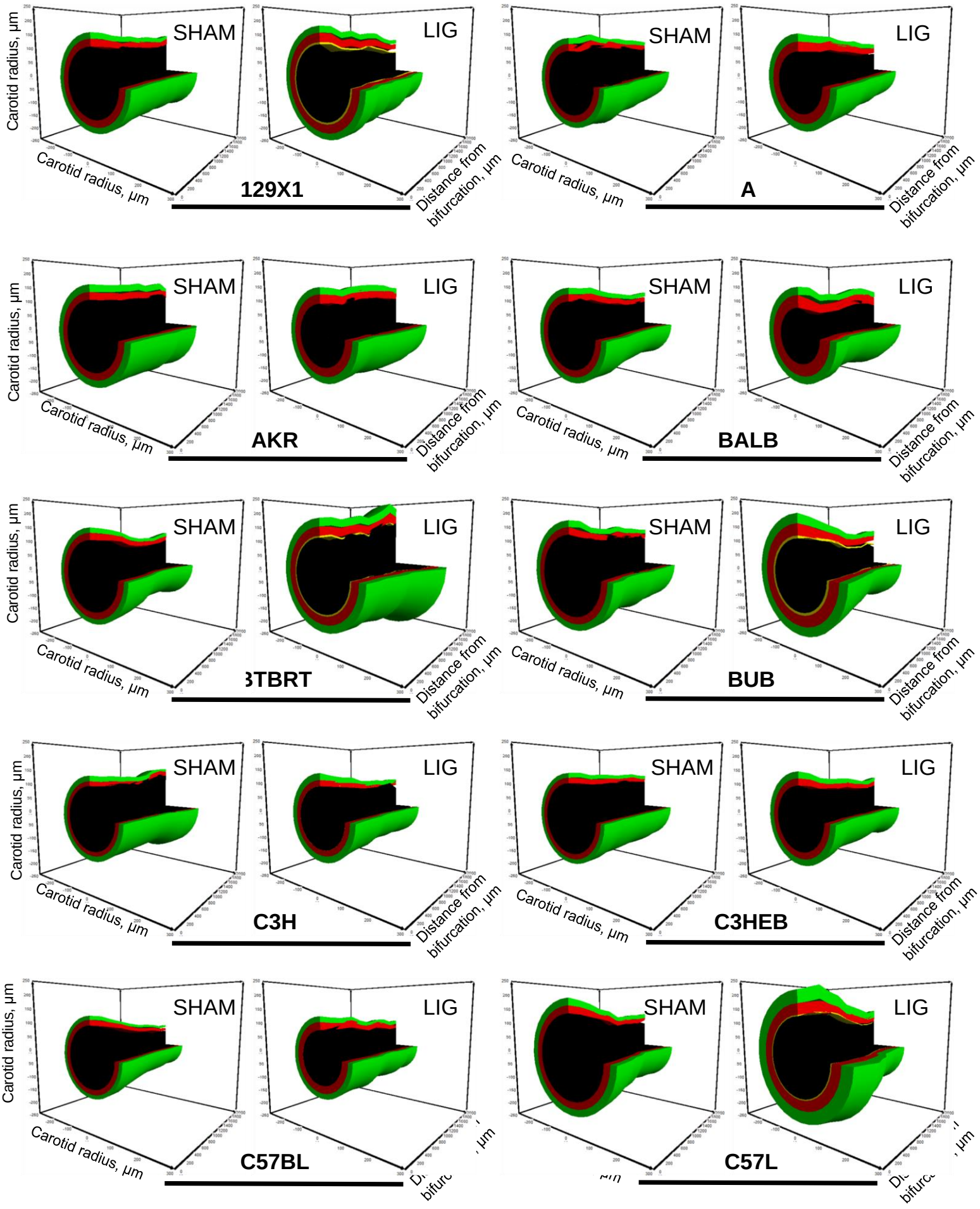
SHAM
LIG
Carotid radius, µm
Distance from bifurcation, µm
129X1
A
AKR
BALB
3TBRT
BUB
C3H
C3HEB
C57BL
C57L

Figure S1

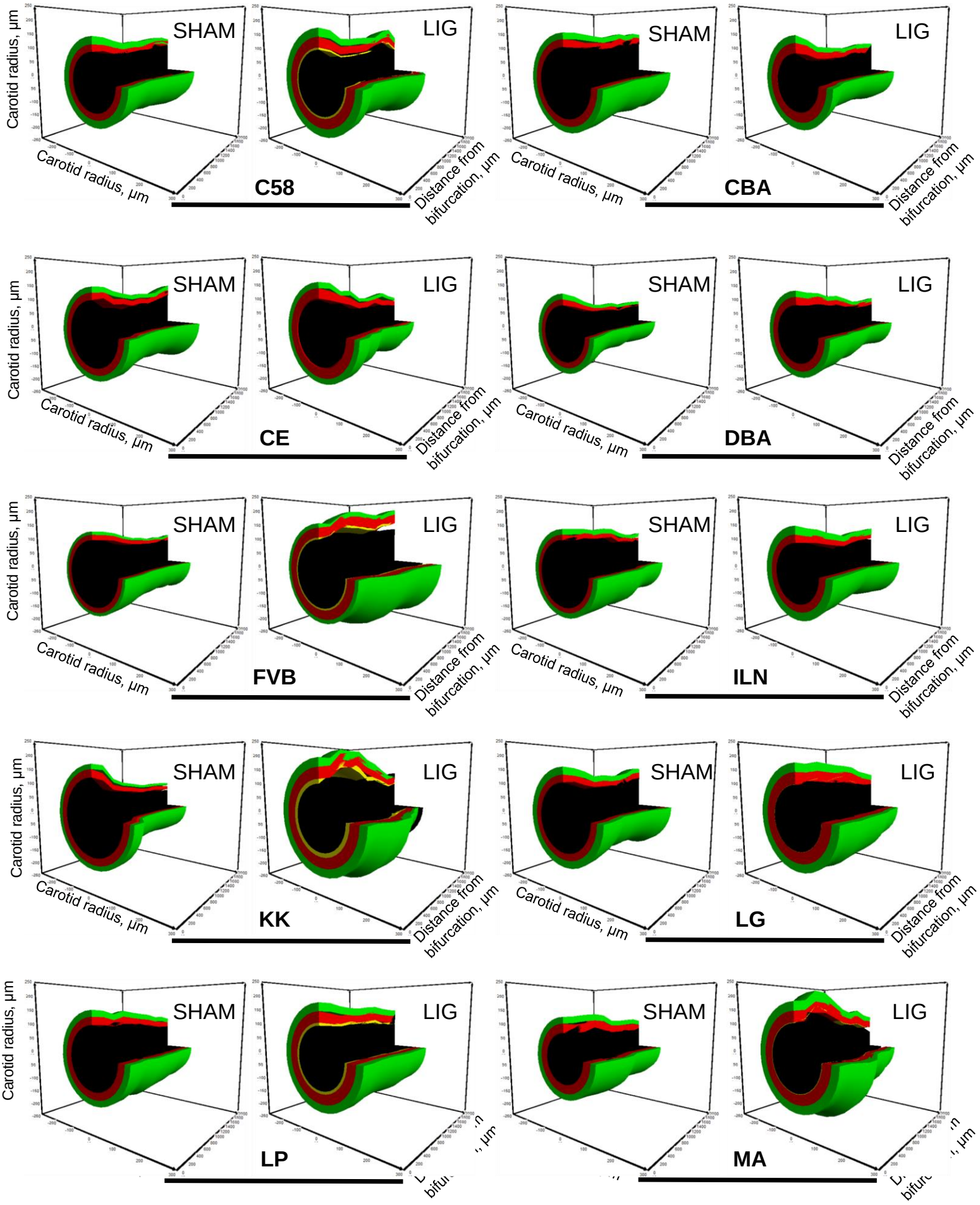
SHAM
LIG
C58
CBA
CE
DBA
FVB
ILN
KK
LG
LP
MA
Carotid radius, µm
Distance from bifurcation, µm

Figure S2

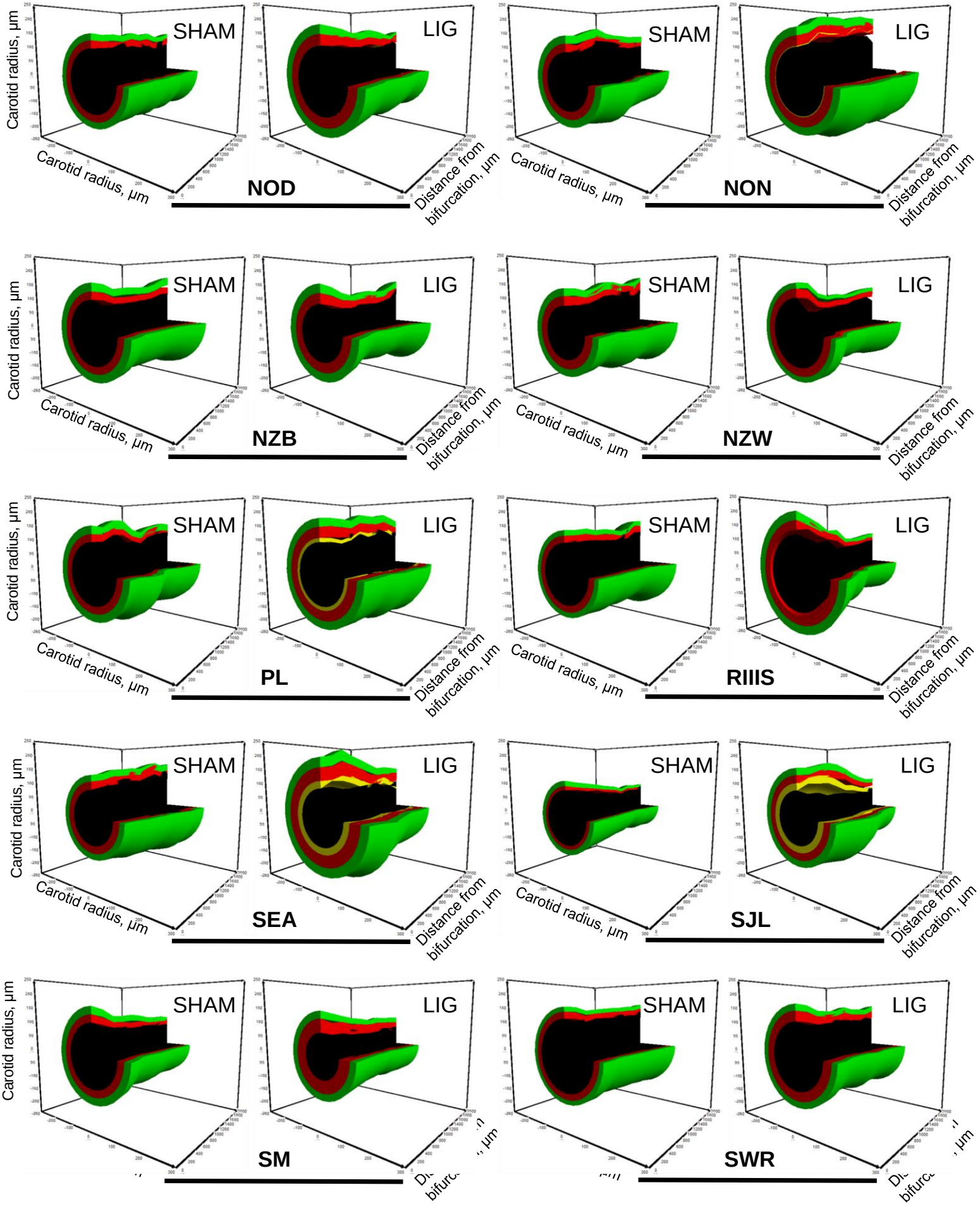
SHAM
LIG
NOD
NON
NZB
NZW
PL
RIIIS
SEA
SJL
SM
SWR
Carotid radius, µm
Distance from bifurcation, µm

Figure S3

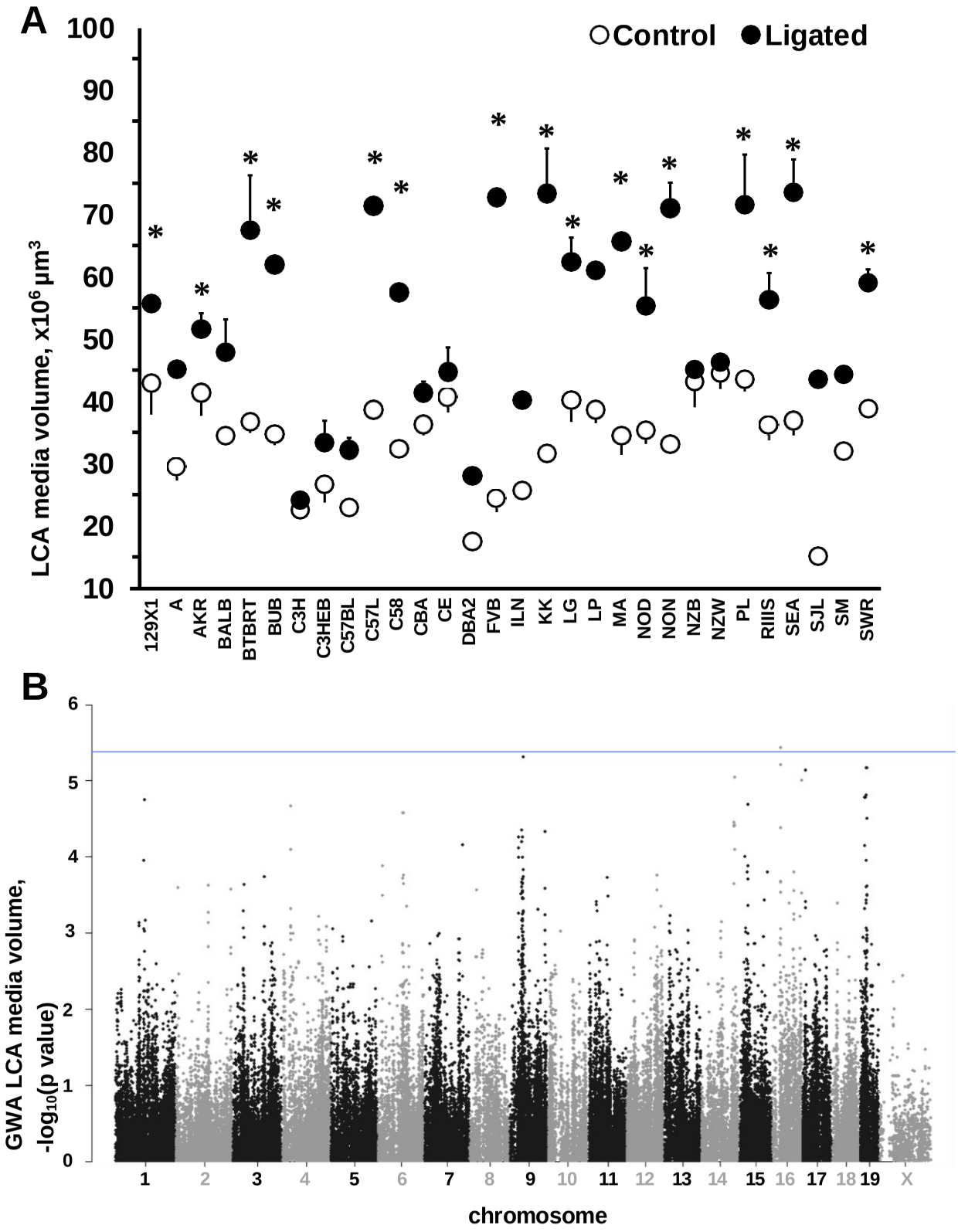
A
Control
Ligated
LCA media volume, x10^6 µm^3
100
90
80
70
60
50
40
30
20
10
129X1
A
AKR
BALB
BTBRT
BUB
C3H
C3HEB
C57BL
C57L
C58
CBA
CE
DBA2
FVB
ILN
KK
LG
LP
MA
NOD
NON
NZB
NZW
PL
RIIIS
SEA
SJL
SM
SWR
B
GWA LCA media volume,
-log10(p value)
6
5
4
3
2
1
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
X
chromosome

Figure S4

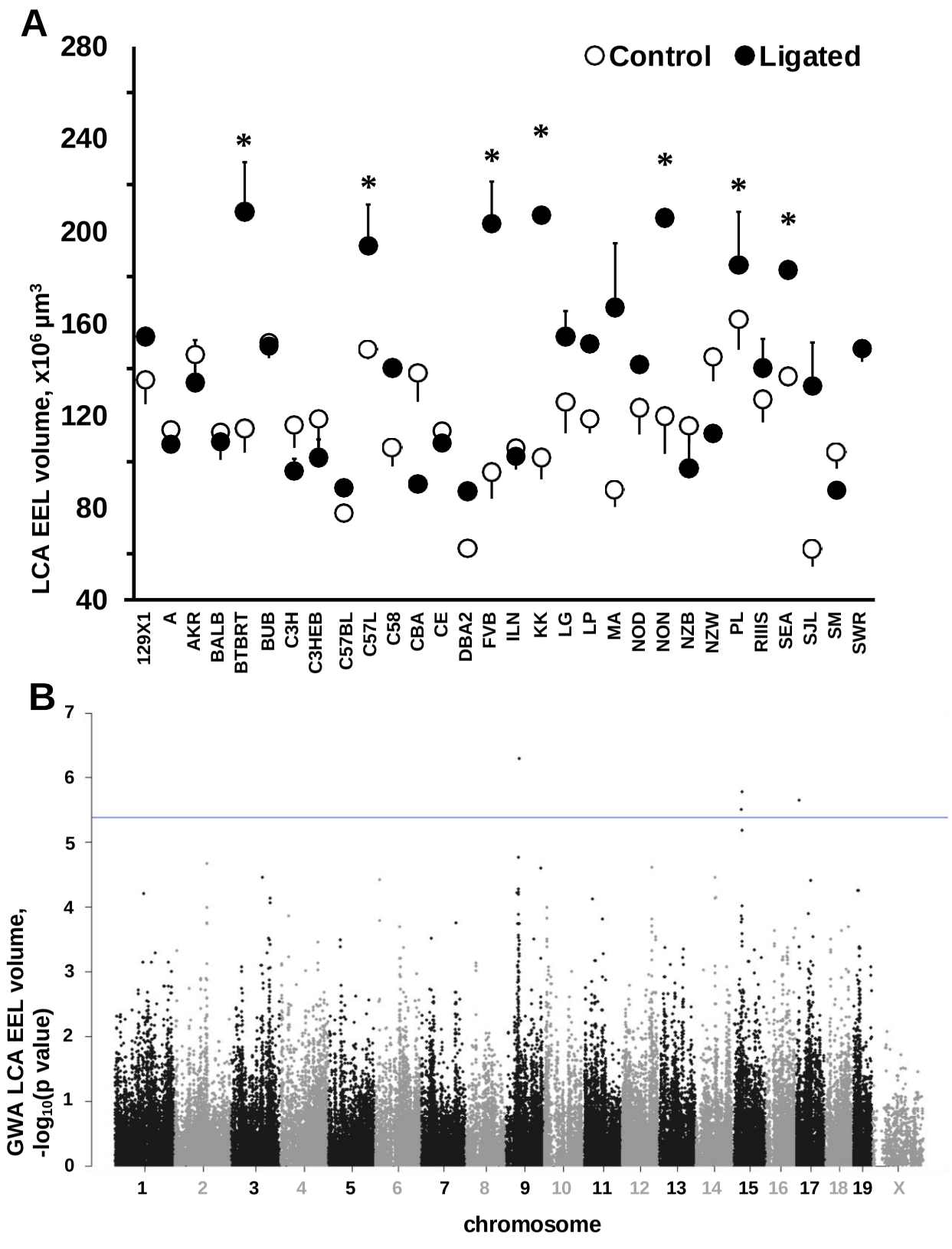
A
Control
Ligated
LCA EEL volume, x10⁶ µm³
280
240
200
160
120
80
40
129X1
A
AKR
BALB
BTBRT
BUB
C3H
C3HEB
C57BL
C57L
C58
CBA
CE
DBA2
FVB
ILN
KK
LG
LP
MA
NOD
NON
NZB
NZW
PL
RIIIS
SEA
SJL
SM
SWR
B
GWA LCA EEL volume, -log10(p value)
0
1
2
3
4
5
6
7
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
X
chromosome

Figure S5

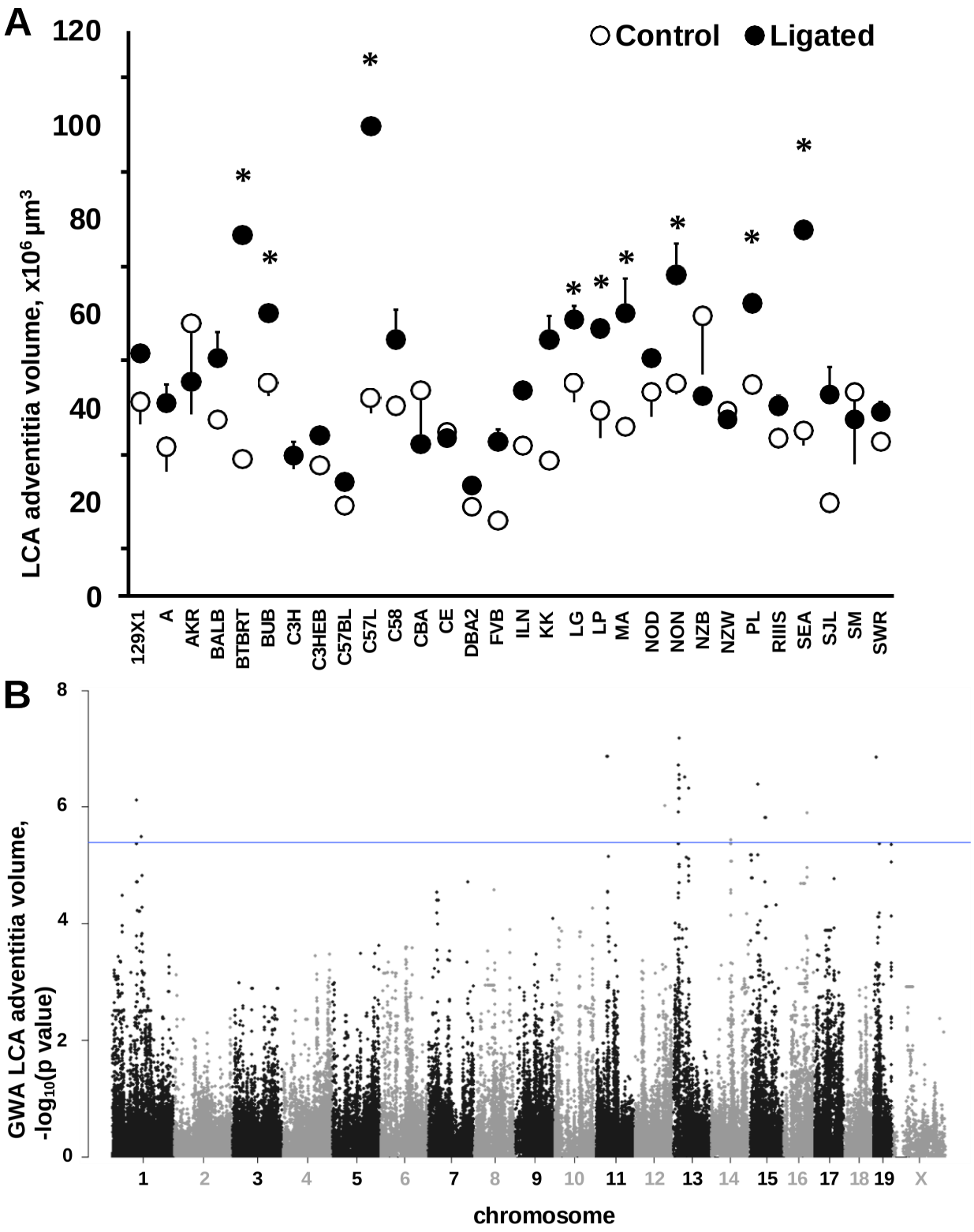
A
Control
Ligated
LCA adventitia volume, x10⁶ µm³
0
20
40
60
80
100
120
129X1
A
AKR
BALB
BTBRT
BUB
C3H
C3HEB
C57BL
C57L
C58
CBA
CE
DBA2
FVB
ILN
KK
LG
LP
MA
NOD
NON
NZB
NZW
PL
RIIIS
SEA
SJL
SM
SWR
B
GWA LCA adventitia volume,
-log10(p value)
0
2
4
6
8
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
X
chromosome

Figure S6

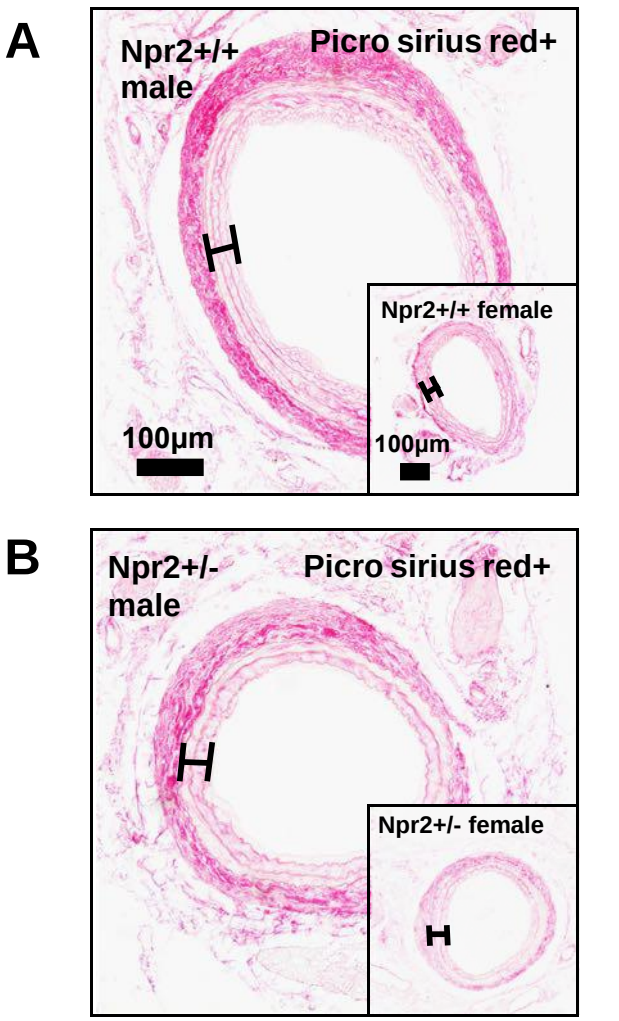
A
Npr2+/+
male
Picro sirius red+
H
100μm
Npr2+/+ female
H
100μm
B
Npr2+/-
male
Picro sirius red+
H
Npr2+/- female
H
C
Picro sirius red IM, %
25
20
15
10
5
0
Npr2+/+
Npr2+/-
*
†
Males
Females

Figure S7