Inhibition of Lysyl Oxidase with β-aminopropionitrile Improves Venous Adaptation after Arteriovenous Fistula Creation

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ABSTRACT

Background: The arteriovenous fistula (AVF) is the preferred hemodialysis access for end-stage renal disease (ESRD) patients. Yet, establishment of a functional AVF presents a challenge, even for the most experienced surgeons, since postoperative stenosis frequently occludes the AVF. Stenosis results from the loss of compliance in fibrotic areas of the fistula which turns intimal hyperplasia into an occlusive feature. Fibrotic remodeling depends on deposition and crosslinking of collagen by lysyl oxidase (LOX), an enzyme that catalyzes the deamination of lysine and hydroxylysine residues, facilitating intra/intermolecular covalent bonds. We postulate that pharmacological inhibition of lysyl oxidase (LOX) increases postoperative venous compliance and prevents stenosis in a rat AVF model.

Methods: LOX gene expression and vascular localization were assayed in rat AVFs and human pre-access veins, respectively. Collagen crosslinking was measured in humans AVFs that matured or failed, and in rat AVFs treated with β-aminopropionitrile (BAPN), an irreversible LOX inhibitor. BAPN was either injected systemically or delivered locally around rat AVFs using nanofiber scaffolds. The major endpoints were AVF blood flow, wall fibrosis, collagen crosslinking, and vascular distensibility.

Results: Non-maturation of human AVFs was associated with higher LOX deposition in pre-access veins (N=20, P=0.029), and increased trivalent crosslinks (N=18, P=0.027) in human AVF tissues. Systemic and local inhibition of LOX increased AVF distensibility, while reducing wall fibrosis and collagen crosslinking in rat fistulas.
**Conclusions**: Our results demonstrate that BAPN-mediated inhibition of LOX significantly improves vascular remodeling in experimental fistulas.
INTRODUCTION

A functional hemodialysis access is the lifeline for ~500,000 end-stage renal disease (ESRD) patients in the United States Foundation (1). The preferred access, the arteriovenous fistula (AVF), created by vein-to-artery anastomosis in the arm (2), incites a complex biological transformation that should yield a superficial vessel with a thickened wall and increased luminal area capable of supporting blood flow over 600 mL/min after maturation (3). However, postoperative venous stenosis stymies maturation, increasing patient morbidity (thrombosis, revision, new AVF) and healthcare costs (4). AVF failure rates have remained unacceptably high (~40%) for decades (4) due greatly in part to the scarce knowledge on the mechanisms underlying AVF stenosis.

The biological processes leading to the formation and progression of stenotic lesions in AVFs have only begun to be elucidated. We have recently disclosed a link between postoperative fibrosis and non-maturation, especially when combined with intimal hyperplasia (IH) (5). The circumferential orientation of collagen fibers around the lumen is also predictive of nonmaturation, suggesting that not only the degree but the quality of medial fibrosis plays a role in venous remodeling (5). Whether the biomechanical characteristics of the postoperative fibrosis determines venous compliance after fistula creation remains unknown.

Lysyl oxidase (LOX) catalyzes the deamination of lysine and hydroxylysine residues, facilitating intra/intermolecular covalent crosslinking (6). Collagen and elastin, the main substrates of LOX, likely govern vascular mechanical properties after fistula
creation (7). In the case of collagen, LOX forms divalent (immature) crosslinks between two adjacent triple-helices, and indirectly promotes the spontaneous (mature) cross-linking with a third triple helix (8). Collagen tensile strength relies upon LOX-mediated crosslinking (9). Hyperactive LOX is associated with hypoxia-induced metastasis (10, 11), myocardial fibrosis (12), and pulmonary hypertension (13). LOX inhibition has been proposed as a pharmacological approach to treat fibrosis (7). However, it is unknown whether dysregulated LOX activity leads to stenosis in failed AVFs. We hypothesize that systemic and local inhibition of LOX with β-aminopropionitrile (BAPN), a naturally occurring LOX inhibitor, promotes adaptive remodeling in experimental AVFs by reducing collagen crosslinking. Systemic administration of BAPN has dose-limiting side effects in humans (i.e., lathyrysm) (14, 15). Therefore, a local delivery method will be tested to achieve high drug concentrations in the fistula with minimal systemic toxicity.

MATERIALS AND METHODS

*Detailed experimental methods are provided in the Supplemental Materials and Methods.*

*Human Veins and AVFs*

Paraffin-embedded cross-sections of pre-access veins (N=20, 10 matured and 10 failed after AVF creation) from patients who received a planned two-stage upper arm AVF at Jackson Memorial Hospital or the University of Miami (UM) were randomly selected from the UM Vascular Biobank. In addition, 18 independent AVF specimens (8 matured and 10 failed) preserved in *RNA*later (Qiagen) and collected at the time of
second-stage surgery were randomly obtained from the biorepository. The study was performed according to the ethical principles of the Declaration of Helsinki and regulatory requirements at both institutions. The ethics committee and Institutional Review Board at the University of Miami approved the study.

*Rat model of AVF maturation*

Sprague Dawley rats (280-320 grams) were purchased from Envigo (Indianapolis, IN). AVFs were created by an end-to-side anastomosis of the epigastric vein to the nearby femoral artery (16). Ninety-six rats of both sexes were randomly allocated to experimental groups for molecular analysis (n=18), systemic BAPN and vehicle administration (n=42), and local drug delivery (n=36).

*Scaffold fabrication and characterization*

Scaffolds were fabricated using an electrospinning apparatus.

*Mass spectrometry*

Cumulative BAPN release was quantified with a direct infusion/ triple quadrupole mass spectrometer (TSQ) (17) configured in positive mode, 42 eV collision energy, [10,000-150,000 m/z] scan range, and parent-ion scan mode. Immature (hydroxylysinonorleucine [HLNL], dihydroxylysinonorleucine [DHLNL]) and mature
crosslinks (pyridinoline [PYD], deoxypyridinoline [DPD]) were quantified in the venous limb of AVFs as previously described (18).

**Pressure myography**

The proximal and distal venous segments of the AVF were placed on a pair of steel cannulas of a pressure myograph system, DMT Model 110P (Danish Myo Technology, Ann Arbor, MI) and secured with surgical nylon sutures. The circumferential wall strain and stress and incremental elastic modulus ($E_{inc}$) were calculated as previously described (19).

**Immunohistochemistry**

Paraffin-embedded rat AVFs were stained with Masson’s trichrome to quantify wall fibrosis. Vascular LOX and elastin were detected in paraffin embedded venous cross-sections with polyclonal antibodies.

**Gene expression**

Total RNA was isolated from the venous limb of AVFs and reverse-transcribed as previously described (20). Changes in gene expression were assessed using the following TaqMan Gene Expression Assays (Applied Biosystems, Waltham, MA).
Statistics

Statistical analyses were performed using GraphPad Prism version 8 for Windows (GraphPad Software, San Diego, CA). Normally distributed data were compared using unpaired t-tests with Welch’s correction and expressed as mean ± standard deviation (or mean ± standard error of the mean [SEM] where indicated in figures). Non-normally distributed data were compared using the Mann-Whitney test and expressed as median and interquartile range (IQR). A P value < 0.05 was considered significant.

RESULTS

Increased collagen crosslinking in human AVFs that failed

We first evaluated the vascular expression of LOX in 20 randomly selected human pre-access veins used for two-stage upper arm AVF creation by immunohistochemistry (IHC). Half of the veins matured, while the remaining 10 failed to mature after AVF creation. Veins that failed had higher accumulation of LOX in the wall (% LOX+ area) than those that matured successfully (median 5.30 [interquartile range, IQR, 3.63-10.32] vs. 1.69 [0.69-5.04], P=0.029; Fig. 1A-B).

We then quantified collagen crosslinking in AVF tissues collected from an independent group of patients (N=18) at the time of second-stage surgery. Eight of these AVFs matured and underwent a standard transposition for superficialization during the second-stage procedure, while 10 AVFs failed and required a short
transposition, ligation, or arteriovenous graft (AVG) extension. The time from AVF creation to postoperative sample collection was similar between AVFs that matured or failed (mean 74.4 ± SD 27.1 vs. 66.7 ± 28.5 days, respectively; P=0.57). AVFs that failed had significantly higher mature crosslinking density than those that matured (0.061 [0.039-0.089] vs. 0.041 [0.025-0.045] µmol per mg of dry weight, P=0.027), and a trend toward increased immature density (1.29 ± 0.56 nmol/mg of dry weight in AVFs that failed vs. 0.87 ± 0.29 in those that matured, P=0.064; Fig. 1C-D). These results support the hypothesis that increased LOX activity during AVF remodeling is associated with access failure.

**AVF creation affects expression of extracellular matrix genes**

The rodent AVF model enables mechanistic studies of AVF remodeling (16). We utilized a rat AVF model to examine the postoperative changes in mRNA expression of *Lox* and *Lox*-like (*LoxL*) genes. *Lox* mRNA was significantly upregulated 5 days post-surgery compared to the expression in the vein at the time of AVF creation (fold change [FC] 2.81 ± 0.92 over baseline, P<0.01; Fig. 2). The AVF portrayed pronounced wall thickening at 20 days after surgery (Supplementary Fig. 1A), representing the adaptation of the vein to arterial circulation. Lysyl oxidase accumulated in cells of the media and adventitia at this time point after AVF creation (Supplementary Fig. 1B).

We then analyzed the mRNA expression pattern of extracellular matrix (ECM) genes and remodeling factors after AVF surgery (Supplementary Fig. 2). Matrix metalloproteases (MMP), fibronectin, elastin, and collagens were upregulated after
surgery. MMP-2 and MMP-9 were upregulated 2.3- and 300-fold, respectively, at postoperative day 5. The alpha 1 chains of collagens type I and III had an increase in expression that peaked at day 5, while elastin increased linearly over 33 days. These findings establish a temporal framework for LOX-targeted interventions.

**Systemic BAPN improves AVF remodeling in rats**

We tested whether inhibition of LOX improved vessel remodeling and AVF outcomes at 21 days post-surgery in the rat model. Systemic inhibition of LOX using BAPN improved blood flow compared to vehicle controls (23.40 ± 10.50 vs. 10.33 ± 4.27 mL/min, P=0.047) (Fig. 3A), and significantly reduced fibrosis (as % of wall area) in the AVF wall (10.42 ± 3.38 vs. 23.72 ± 11.88 %, P=0.010; Fig. 3B-C, Supplementary Fig. 3A). BAPN treatment reduced immature collagen crosslinking compared to vehicle controls (0.42 ± 0.12 vs. 1.19 ± 0.33 µmol/mg of dry weight, P<0.01; Fig. 3D), and showed a trend toward decreasing mature crosslinking (0.10 ± 0.03 vs. 0.21 ± 0.15 µmol/mg of dry weight, P=0.12; Fig. 3E). Interestingly, systemic inhibition of LOX decreased wall thickness in the fistula compared to the vehicle control (Supplementary Fig. 3B), suggesting an effect on smooth muscle cell (SMC) proliferation and/or migration. However, in contrast to the reduction of wall fibrosis (Fig. 3C), the treatment had no effect on elastin content (Supplementary Fig. 4A-B). Treated AVFs also had more distensibility (% deformation at increasing intraluminal pressures; Fig. 3F) and elasticity, as indicated by a lower value of incremental Young’s modulus (E_{inc}; Fig. 3G). These results suggest that aberrant LOX activity contributes to maladaptive venous
remodeling. Furthermore, it demonstrates the efficacy of systemic BAPN-mediated inhibition of LOX in improving the biomechanical performance of experimental AVFs.

**Scaffold fabrication and characterization**

We developed an electrospun scaffold that can be applied to the venous adventitia intraoperatively for local, sustained BAPN delivery to the AVF to improve efficacy and minimize off-target effects. After systematic *in vitro* characterization of fabricated scaffolds (data not shown), we identified process (voltage, flow rate, needle diameter, etc.) and solution (polymer concentration, drug loading) parameters that yielded fibers with pristine nanoscale morphology and adequate degradation and release kinetics (Fig. 4A-B and Supplementary Fig. 5). The average fiber diameter for BAPN and control scaffolds was 185 ± 151 and 392 ± 132 nm (P<0.01), respectively. In vitro, both BAPN and control scaffolds degraded entirely in 60 days (Fig. 4A). BAPN scaffolds degraded progressively losing 50% of their mass in 30 days, while control scaffolds had minimal degradation until day 40. Both scaffolds had degraded beyond detection by day 21 postop in vivo (data not shown). Drug-loaded scaffolds liberated 20% of BAPN content on day 1 in vitro, followed by a progressive release of additional 20% over the next 30 days (Fig. 4B).
Local BAPN delivery promotes adaptive remodeling in experimental AVFs

We wrapped AVFs intraoperatively with BAPN or vehicle-loaded scaffolds to test the feasibility of local LOX inhibition as prophylactic therapy. BAPN scaffolds significantly decreased wall fibrosis (34.79 ± 3.83 vs. 47.17 ± 8.65 %, P=0.029) and showed a nonsignificant trend toward higher blood flow compared to controls (34.03 ± 17.32 vs. 24.80 ± 7.60 mL/min, P=0.28; Fig. 4C-D) at day 21 postop. Of note, irrespective of the presence of drug, the perivascular scaffold modified AVF remodeling in this model, since both groups of animals had higher blood flows and fibrosis compared to the systemic BAPN treatment and control groups (Fig. 3A-C). AVFs wrapped with control scaffolds had more elastin content than those treated with BAPN locally and both systemic experimental groups, suggesting a protective effect of the scaffold on elastin degradation (Supplementary Fig. 4B). In contrast to the systemic BAPN treatment, drug-loaded scaffolds significantly decreased elastin deposition, likely as a result of higher effective drug concentrations. Interestingly, there was no significant difference in wall thickness between the local experimental groups, but both showed lower thickness than the systemic control and BAPN arms (Supplementary Fig. 3B). Again, these results suggest an effect of the scaffold by itself on AVF remodeling.

As with the systemic treatment (Fig. 3D-E), BAPN decreased immature crosslinking compared to controls (0.82 ± 0.32 vs. 1.28 ± 0.29 µmol per mg of dry weight, P=0.025; Fig. 4E), and showed a trend toward reduced mature crosslinking (0.07 [0.05-0.13] vs. 0.15 [0.11-0.38] µmol per mg of dry weight, P=0.065; Fig. 4F). Furthermore, consistent with systemic administration of BAPN (Fig. 3F-G), local treatment improved biomechanical properties in the rat AVF model (Fig. 4G-H). These
results suggest that local BAPN delivery using perivascular scaffolds is an effective option to improve AVF maturation.

DISCUSSION

We recently established vascular fibrosis as a hallmark of AVF failure (5). However, whether the posttranslational modification of newly synthesized collagen determines AVF compliance is uncertain. LOX mediates the oxidation of ε-amino groups of hydroxylysine residues to facilitate crosslinking among collagen bundles (6). Since LOX increases vessel stiffness and reduces elastic compliance in arteries (21, 22), we hypothesized that dysregulated LOX activity after AVF creation promotes venous stenosis. Herein, we identified a link between increased LOX deposition in the wall of pre-access human veins and non-maturation of the AVFs. Collagen was more crosslinked in AVFs that failed than in those achieving maturation. LOX and its ECM substrates were rapidly upregulated after AVF creation in rats. We also demonstrated that systemic and local administration of BAPN reduced collagen crosslinking and promoted adaptive remodeling in rat fistulas. These experiments confirm the noxious effect of LOX during AVF remodeling, paving a new therapeutic avenue to prevent AVF failure.

The higher levels of vascular LOX in human pre-access veins that failed vs. those that matured, and of collagen crosslinking in postoperative AVF tissues that failed compared to fistulas that remodeled successfully, suggest that collagen fibrillogenesis is a critical process in determining AVF compliance and outward remodeling early after AVF creation. Despite the self-assembly properties of collagen molecules in vitro,
collagen fibril formation is heavily regulated in tissues by procollagen N- and C-terminal proteinases, fibronectin, vascular integrins, non-enzymatic collagen glycation, and crosslinking enzymes such as LOX (23, 24). Specifically, LOX catalyzes the divalent (immature) bridging of two adjacent collagen triple-helices. Within hours, this initial reaction promotes the spontaneous (mature) crosslinking with a third collagen triple helix in a process that continues for months (8). AVFs that failed have significantly higher mature crosslinking than those that matured, and only an increasing trend in immature crosslink density, likely as a result of the continuous modification of collagen fibers during the 2-3 months that elapsed from AVF creation to sample collection.

Collagen crosslinking is critical for fibril stability, vascular integrity, and function. However, hyperactive LOX may lead to vascular stiffness and is known to promote intimal hyperplasia (21, 22, 25). Our group previously showed that collagen deposition increases significantly after AVF creation in humans, and that increased postoperative medial fibrosis was associated with non-maturation in a cohort of 115 patients (5). Altogether, the current data suggest that in addition to the degree of postoperative medial fibrosis, collagen crosslinking is an exacerbating factor that contributes to impaired maturation.

Our rat AVF model also demonstrated upregulation of LOX after surgery, along with genes encoding for collagen I and III, the main collagen types involved in vascular remodeling (26, 27). Sustained fibronectin upregulation after AVF creation in rats also supports an increase in postoperative fibrillogenesis. These findings are in line with earlier studies describing increased LOX expression as a requisite for ECM stabilization after arterial injury in rats (28). In the latter model, elevated LOX activity was associated
with acute constrictive remodeling as a result of excessive collagen and elastin crosslinking (28).

We then postulated that inhibition of LOX using BAPN, a natural irreversible inhibitor of LOX (29) and LOXL (14, 30), could prove beneficial for postoperative remodeling in the rat AVF model. Systemic BAPN treatment significantly reduced immature crosslinking in AVFs and showed a strong trend toward decreased mature crosslinking. Considering that mature crosslinking of newly synthesized collagen occurs over time, it is possible that a significant change between the control and treatment groups is not detectable at 21 days after AVF creation. Importantly, systemic inhibition of LOX with BAPN during the period of maturation significantly improved the biomechanical performance of AVFs, as indicated by an increase in venous distensibility and elasticity. This increased in elasticity occurred in the absence of changes in elastin content by systemic BAPN, suggesting that elasticity in fistulas is likely more dependent on elastin crosslinking and fiber organization than on protein content. Elastin is not only a substrate of LOX, but its expression is also regulated by LOX at the transcriptional level (31). The role of elastin in AVF remodeling is not clear at the moment. Animal models suggest that lower amounts of elastin enhance outward remodeling of the fistula (32). However, a clinical trial with local elastase improved maturation in radiocephalic AVFs but not in brachiocephalic fistulas (33).

Electrospun nanofibers have been widely used as vascular biomaterials due to their structural and mechanical properties which closely resemble the vascular ECM (34-37). We developed an electrospun scaffold for perivascular delivery of BAPN to increase the effective drug concentration while minimizing off-target effects (38). BAPN-
loaded nanofibers progressively eroded over 60 days in vitro, while control scaffolds had negligible mass loss until day 40, consistent with bulk degradation (39). BAPN scaffolds had a biphasic drug release profile with a drastic burst followed by sustained release. Surface erosion and burst release of BAPN scaffolds can be attributed to the presence of the hydrophilic BAPN at the fiber interface, which facilitates hydrolysis at the fiber surface and early release (39-42). This pharmacokinetic profile was effective at decreasing immature crosslinking and fibrosis, and improving biomechanical properties, in agreement with the systemic BAPN regimen. However, further characterization of the effects of PLGA scaffolds on AVF remodeling is needed, since both scaffold treatment groups had higher fibrosis and blood flow than the systemic arms, despite similar crosslinking and biomechanical properties between the corresponding drug to drug and vehicle to vehicle comparisons. In fact, control scaffolds resulted in higher elastin content than drug-loaded scaffolds and both systemic experimental groups, suggesting a protective effect of the empty scaffold on elastin degradation. Unlike the systemic treatment, the higher effective drug concentration achieved with local BAPN delivery decreased elastin content in the drug-loaded group, in agreement with published studies in which BAPN annulled the positive action of LOX on the elastin promoter (31). Despite less elastin, AVFs treated with drug-loaded scaffolds had higher elasticity than controls, again indicating than elasticity in fistulas in dependent on other factors besides elastin content. Another interesting effect observed in both scaffold treatment groups was reduced AVF wall thickness with respect to the systemic experimental arms. This effect may explain the higher blood flows observed compared to the latter, and is in line with published work with drug-free PLGA scaffolds in porcine vein grafts (43, 44).
Overall, our results suggest that periadventitial scaffolds alter adaptive remodeling via multiple mechanisms, including a potential contribution of “external stenting” (44-50). It has been proposed that bioabsorbable, nonrestrictive scaffolds improve vein remodeling by 1) modulating hemodynamic forces, 2) increasing vascularization of the adventitia, and/or 3) promoting outward migration (as opposed to inward remodeling) of SMCs in response to inflammatory cell infiltration of the scaffold (51).

One question that remains is whether the different types of collagen crosslinks play distinct roles in AVF remodeling. It would be difficult to separate their effects since mature crosslinks depend on the occurrence of immature ones. However, it is tempting to hypothesize that mature crosslinks are more restrictive for AVF biomechanics given their interactions with a third collagen triple helix. On the other hand, different levels of collagen organization may have distinct effects on de novo collagen deposition, binding of LOX and other ECM remodeling enzymes, sequestration of growth factors, migration of cells, etc. Inhibition of immature crosslinks was sufficient to improve biomechanics in the rat AVF without significantly changing mature crosslinking. Future studies where AVFs biomechanics are studied at increasing time points after creation may allow for a better evaluation of the roles of mature vs. immature crosslinking in remodeling.

The limitations of the study include the use of a small animal model for the study of AVF hemodynamics, the lack of specificity of BAPN which also targets LOXL enzymes (29, 52-56), the lack of statistical power to evaluate sex related effects, and the use of a non-unidirectional scaffold that allows drug release to perivascular tissues. Addressing these limitations in the future may prove instrumental to the development of novel preventive therapies for early AVF failure. Despite these issues, we demonstrated
the efficacy of systemic and perivascular BAPN-mediated inhibition of LOX in improving the biomechanical properties of experimental AVFs. Given the observed differences in collagen crosslinking between human AVFs that failed and those that matured, our results advocate that inhibition of LOX is an effective method to optimize outward remodeling after AVF creation.

Disclosures
L. Salman reports Research Funding: Transonics Inc., Roach funds, Albany Medical Center; Patents and Inventions: I have a patent application for the use of 4-Methylumbelliferone in Diabetic Kidney Disease. The application is pending review.; Other Interests/Relationships: American Society of Diagnostic and Interventional Nephrology, American Society of Nephrology, Renal Physician Association, Data Safety Monitoring Board – Phraxis. O. Velazquez reports Scientific Advisor or Membership: NIH. All remaining authors have nothing to disclose.

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Supplemental Materials

- Supplemental Materials and Methods
- Supplemental References
- Supplementary Figure 1. Postoperative venous remodeling and LOX localization in the rat femoral-epigastric AVF.
- Supplementary Figure 2. Postoperative gene expression (GE) of vascular remodeling genes in the venous limb of the rat femoral-epigastric AVF.
- Supplementary Figure 3. Fibrosis and wall thickness in BAPN-treated and control AVFs.
- Supplementary Figure 4. Elastin content in BAPN-treated and control AVFs.
- Supplementary Figure 5. Scanning electron microphotograph of BAPN-loaded PLGA nanofibers.
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FIGURE LEGENDS

Figure 1. Increased lysyl oxidase (LOX) deposition and collagen crosslinking are associated with nonmaturation of human arteriovenous fistulas (AVFs). A. Representative anti-LOX immunohistochemistry in pre-access upper arm veins that matured or failed after AVF creation. B. Quantification of the LOX-positive wall area in cross-sections of pre-access veins that matured (N=10) or failed (N=10). C-D. Immature (C) and mature (D) crosslinking density in human upper arm AVF tissues that matured (N=8) or failed (N=10), collected at the time of second-stage transposition surgery. Divalent immature crosslinks hydroxylysinonorleucine (HLNL) and dihydroxylysinonorleucine (DHLNL), and trivalent mature crosslinks pyridinoline (PYD) and deoxypyridinoline (DPD) were quantified in UPLC-ESI-MS/MS. Immature crosslinking density is expressed as nmol per mg of dry weight of tissue, while mature density is presented as µmol per mg of dry weight.

Figure 2. Lysyl oxidase (LOX) is upregulated in experimental AVFs after surgery. Gene expression (GE) of LOX and lysyl oxidase-like enzymes (LOXL) in the venous limb of rat AVFs harvested 5 days post-surgery. mRNA expression was normalized with respect to GAPDH and expressed as fold change vs. the contralateral vein at the time of AVF creation. Bars represent the mean ± SEM, n=3-6 per group.

Figure 3. Systemic inhibition of lysyl oxidase (LOX) with BAPN improves the biomechanical performance of experimental AVFs. A. AVF blood flow in BAPN-
treated and control rats at 21 days post surgery. Bars represent the mean ± SEM (n=5-6 per group). B. Representative Masson’s trichrome stained venous sections from BAPN-treated and control AVFs. Fibrosis stained blue while cells are red. C. Fibrosis quantification (as % of wall area) in Masson’s trichrome stained sections from both experimental groups. Bars represent the mean ± SEM (n=9 per group). D-E. Immature (D) and mature (E) crosslinking density in the venous limb of BAPN-treated and control AVFs as determined by UPLC-ESI-MS/MS (n=5-6 per group). Crosslinking density is expressed as µmol per mg of dry weight of tissue. F-G. AVF distensibility (F) and elasticity (G) assessed in a pressure myograph under Ca$^{2+}$-free conditions. Distensibility is expressed as % deformation vs. the external diameter of the vein at 3 mmHg. Elasticity is based on the incremental Young’s modulus ($E_{inc}$), in which lower $E_{inc}$ values are indicative of more elastic vessels. Dots represent the mean ± SEM (n=5-6 per group).

Figure 4. Local BAPN delivery improves the biomechanical properties of experimental AVFs. A. Degradation profiles of BAPN- and vehicle-loaded scaffolds over 60 days in vitro. B. Cumulative BAPN release from PLGA scaffolds over 30 days in vitro. C. AVF blood flow in BAPN-treated and control rats at 21 days post surgery. Bars represent the mean ± SEM (n=5-6 per group). D. Fibrosis quantification (as % of wall area) in Masson’s trichrome stained venous sections from BAPN-treated and control AVFs. Bars represent the mean ± SEM (n=5-6 per group). E-F. Immature (E) and mature (F) crosslinking density in the venous limb of AVFs treated with BAPN and vehicle as quantified by UPLC-ESI-MS/MS (n=6 per group). Crosslinking density is
expressed as µmol per mg of dry weight. **G-H.** AVF distensibility (G) and elasticity (H) assessed in a pressure myograph under Ca^{2+}-free conditions. Distensibility is expressed as % deformation vs. the external diameter of the vein at 3 mmHg. Elasticity is based on the incremental Young's modulus (E_{inc}), in which lower E_{inc} values are indicative of more elastic vessels. Dots represent the mean ± SEM (n=4-6 per group).
Figure 1

A

B

C

D

HLNL + DHLNL density

P = 0.064

PYD + DPD density

P = 0.027

Matured
Failed

Matured
Failed

% LOX+ Area

P = 0.029
Figure 2

GE (Fold Change vs. Vein)

Lox  LoxL1  LoxL2  LoxL3  LoxL4

P<0.01
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MATERIALS AND METHODS

Human Veins and AVFs

Paraffin-embedded cross-sections of pre-access veins (N=20, 10 matured and 10 failed after AVF creation) from patients who received a planned two-stage upper arm AVF at Jackson Memorial Hospital or the University of Miami (UM) were randomly selected from the UM Vascular Biobank. In addition, 18 independent AVF specimens (8 matured and 10 failed) preserved in RNaLater (Qiagen) and collected at the time of second-stage surgery were randomly obtained from the biorepository. All AVFs were created by a single vascular surgeon (M.T.). Anatomic non-maturation was defined as an AVF that never achieved an internal diameter ≥ 6 mm. The time from access creation to second-stage surgery was similar between AVFs that matured and those that failed (74.4 ± 27.1 vs. 66.7 ± 28.5 days, respectively; P=0.57), and both subgroups were comparable in terms of demographics and comorbidities. The second-stage surgery consisted of a standard AVF transposition for AVFs that matured or a salvage procedure (short transposition, ligation, or AVG extension) for those that failed. Enrolled patients were consented before sample collection at the corresponding surgeries. The study was performed according to the ethical principles of the Declaration of Helsinki and regulatory requirements at both institutions. The ethics committee and Institutional Review Board at the University of Miami approved the study.

Rat model of AVF maturation

Sprague Dawley rats (280-320 grams) were purchased from Envigo (Indianapolis, IN). AVFs were created by an end-to-side anastomosis of the epigastric
vein to the nearby femoral artery (1). Ninety-six rats of both sexes were randomly allocated to experimental groups. Molecular analysis of AVFs was performed at 0, 5, 20, and 33 days post-surgery (n=18). For systemic drug administration, rats were given a daily intraperitoneal injection of BAPN (100 mg/kg; Sigma-Aldrich, St. Louis, MO) (n=21) or PBS (vehicle, n=21) on days -2 to 21 post surgery. For local delivery, scaffolds with BAPN (n=18) or PBS (n=18) were wrapped around the epigastric vein immediately after AVF creation. Flow was measured using a flowmeter (Transonic Systems Inc., Ithaca, NY) prior to AVF harvesting at day 21. Rats were euthanized with isoflurane, after which AVFs were removed and placed in ice-cold normal saline for pressure myography, RNAlater for qRT-PCR, formalin for histology, or liquid nitrogen for collagen crosslinking analyses. The overall attrition rate was 4.2%, including 3 animals with postsurgical complications and one animal excluded due to technical reasons (see below).

Two out of the 42 rats used for systemic BAPN/vehicle injections were excluded due to complications with surgical sutures. From the remaining 40, 11 were used for intraoperative flow measurements followed by myography (3 males [M] and 3 females [F] in the vehicle group, 3M and 2F in the BAPN arm), 18 independent animals were used to measure fibrosis (5M and 4F in both experimental arms), and 11 independent rats were used for mass spectrometry (3M and 3F in the vehicle group, 2M and 3F in the BAPN arm).

One rat from the 36 included in the scaffold experiments was euthanized due to excessive postoperative bleeding. From the remaining 35, 11 were used for intraoperative flow measurements (3M and 2F in the vehicle group, 3M and 3F in the BAPN arm). One additional AVF in the vehicle arm was damaged during flow evaluation
and had to be excluded from the subsequent myography experiments. The final set for myography consisted of 2M and 2F in the vehicle arm, and the remaining 3M and 3F in the BAPN group). Eleven independent rats were used to measure fibrosis (2M and 3F in the vehicle group, 3M and 3F in the BAPN arm), and 12 more for mass spectrometry (3M and 3F in both experimental arms).

We did not suffer any attrition in the group of 18 rats used for molecular analyses. The four time points when gene expression was measured (0, 5, 20, and 33 days) included similar numbers of male and female animals: 2M and 1F at time zero, 3M and 3F at 5d, 3M and 3F at 20d, and 2M and 1F at 33d.

*Scaffold fabrication and characterization*

Scaffolds were fabricated using an electrospinning apparatus under the direction of F.M.A. Ester-terminated PLGA (Resomer® RG 504) was purchased from Sigma-Aldrich. PLGA (15% w/v) and drug (10% w/w PLGA) were dissolved in hexafluoroisopropyl alcohol and stirred overnight. PLGA solutions with and without drug were loaded into a 3 mL syringe attached to a blunt 22G stainless steel needle. A 20-kV voltage was applied to the needle. Flow was maintained at 2.0 mL/h using an Aladdin Single-Syringe Pump (World Precision Instruments, Sarasota, FL). Nanofibers were deposited on an aluminum-wrapped collector located 13 cm from the needle tip. Scaffolds were dried overnight in a fume hood to remove any residual solvent. Fiber architecture was analyzed using scanning electron microscopy (SEM) (JEOL Ltd., Tokyo, Japan) after gold sputter-coating. Fiber diameter was determined in ImageJ
by adding scale bars to the width of randomly selected fibers before averaging. For degradation, pre-weighed sections of PLGA scaffolds were placed in 1.5 mL of PBS and incubated in a water bath at 37°C. Scaffolds were then removed from the PBS, lyophilized, and weighed, before incubating in fresh PBS again until the next time point. The “old” PBS was stored for mass spectrometry (MS). Cumulative BAPN release was determined in tandem with degradation experiments. Percent release was calculated as the ratio of BAPN in the supernatant to the initial amount of BAPN in the PLGA scaffold.

**Mass spectrometry**

Cumulative BAPN release was quantified with a direct infusion/ triple quadrupole mass spectrometer (TSQ)(2) configured in positive mode, 42 eV collision energy, [10.000-150.000 m/z] scan range, and parent-ion scan mode. Stock solutions of BAPN (1 mg/mL) and aminoacetonitrile internal standards (IS; 1 mg/mL) were prepared in methanol to establish the Internal Response Factor (IRF), (Equation I). After calculating the IRF, the Amount Specific Compound (SC) of BAPN (m/z=71.42) was determined in relation to the IS (m/z=57.47) at different time points over 60 days (Equation II).

\[
\text{Internal Response Factor} = \frac{\text{Intensity}_{IS} \times \text{Amount}_{SC}}{\text{Amount}_{IS} \times \text{Intensity}_{SC}} \quad (I)
\]

\[
\text{Amount Specific Compound} = \frac{\text{Amount}_{IS} \times \text{Intensity}_{SC} \times \text{IRF}_{SC}}{\text{Intensity}_{IS}} \quad (II)
\]
Immature (hydroxylysinonorleucine [HLNL], dihydroxylysinonorleucine [DHLNL]) and mature crosslinks (pyridinoline [PYD], deoxypyridinoline [DPD]) were quantified as previously described.(3) Briefly, human and rat AVF tissues were lyophilized overnight, reduced with sodium borohydride (NaBH₄) in NaOH, and hydrolyzed overnight with 6N HCl. Samples were lyophilized again and injected (5 µL) into an Acclaim™ Polar Advantage II LC reverse-phase column (Thermo Scientific™). MS was performed with positive electrospray ionization (ESI) with parameters and MRM transitions optimized for immature and mature crosslinks detection.(3) Crosslinks were quantified using the ratio of peak area of epinephrine to pyridoxine as IS. The calibration curves for HLNL, DHLNL, PYD, DPD, and hydroxyproline were established through a linear least-squares regression with a weighing factor of 1/C². Immature and mature crosslinking density is expressed as the amounts of crosslinks per mg of dry weight of tissue.

Pressure myography

Rat AVFs were collected and cleaned of fat at 21 days post-surgery. The proximal and distal segment of the AVF were placed on a pair of steel cannulas (outer diameter: 900 or 1200 µm, depending on fistula size) of a pressure myograph system, DMT Model 110P (Danish Myo Technology, Ann Arbor, MI) and secured with surgical nylon sutures. After the distal segment of the artery was secured, and the vessel length adjusted to eliminate stretch, the intraluminal pressure was raised to 70 mmHg. The mounted vessels were equilibrated for 30 min at 37°C in calcium-free PSS gassed with a mixture of 95% O₂ and 5% CO₂, before reducing the intraluminal pressure to 3 mmHg. To obtain the pressure-diameter curve, the internal and external diameters were
measured while increasing intraluminal pressure in 10 mmHg steps between 3 and 130 mmHg. The diameters were recorded using the DMT MyoView 4 software (Danish Myo Technology). Distensibility was calculated as % deformation at increasing pressures with respect to the external diameter at 3 mmHg. The circumferential wall strain and stress and incremental elastic modulus (E_{Inc}) were calculated as previously described.(4)

**Immunohistochemistry**

Paraffin-embedded rat AVFs were stained with Masson’s trichrome to quantify wall fibrosis. Percent area of fibrosis was calculated in ImageJ using color thresholding. Briefly, images were converted to RGB format and color thresholds on the blue channel were used to segment the blue (collagen) from the red/pink (cells) staining.

For staining of LOX in human and rat AVFs, cross-sections were rehydrated by serially immersing them in xylene, alcohol, and water. Antigen retrieval for LOX staining was performed by boiling slides in 10 mM citrate buffer (pH 6.0) for 20 minutes. Antigen retrieval for elastin was done using proteinase K digestion for 10 minutes at 37°C. Next, sections were blocked for 20 minutes with Tris–Borate saline supplemented with 15% fetal bovine serum (FBS), followed by 3% hydrogen peroxide. Veins and AVFs were incubated with either anti-LOX polyclonal antibody at room temperature for 1 hour (Cat# ab31238, 1:200, Abcam, Cambridge, UK) or anti-elastin polyclonal antibody (Cat# ab23748, 1:200, Abcam) overnight. Bound antibodies were detected using the Dako Universal Link kit (Agilent, Santa Clara, CA), and color was developed with the Dako DAB+ Substrate Chromogen System (Agilent). Nuclei were counterstained with Meyer's
hematoxylin and mounted in Entellan (Sigma-Aldrich). Images were acquired using a VisionTek DM01 digital microscope (Sakura Finetek, Torrance, CA). Operators blinded to the clinical data performed digital processing of images.

**Gene expression**

Gene expression of Lox and LoxL genes was measured in rat AVFs harvested 5 days after surgery. For ECM genes and remodeling factors, tissues were collected at days 5, 20, and 33 after surgery. Samples were immediately stored in RNAlater at −80°C. Total RNA was isolated and reverse-transcribed as previously described.(5) Changes in gene expression were assessed using the following TaqMan Gene Expression Assays (Applied Biosystems, Waltham, MA): *Col1a1*, Rn01463848_m1; *Col3a1*, Rn01437681_m1; *Col5a1*, Rn00593170_m1; *Eln*, Rn01499782_m1; *Fbln5*, Rn00569712_m1; *Fgfr2*, Rn01269940_m1; *Fn1*, Rn00569575_m1; *Gapdh*, Rn99999916_s1; *Lox*, Rn01491829_m1; *Loxl1*, Rn01418038_m1; *Loxl2*, Rn01466080_m1; *Loxl3*, Rn01765241_m1; *Loxl4*, Rn01410872_m1; *Mmp2*, Rn01538170_m1; *Mmp9*, Rn00579162_m1; *Timp1*, Rn00587558_m1; *Timp2*, Rn00573232_m1; and *Timp4*, Rn01459160_m1. Real-time PCR was performed on an ABI Prism 7500 Fast Real-Time PCR System (96-well plate; Applied Biosystems). Relative gene expression was determined using the ΔΔCT method.(6) Gene expression was normalized with respect to GAPDH and expressed as fold change vs. the contralateral vein at the time of AVF creation.
Statistics

Statistical analyses were performed using GraphPad Prism version 8 for Windows (GraphPad Software, San Diego, CA). Normally distributed data were compared using unpaired t-tests with Welch’s correction and expressed as mean ± standard deviation (or mean ± standard error of the mean [SEM] where indicated in figures). Non-normally distributed data were compared using the Mann-Whitney test and expressed as median and interquartile range (IQR). A P value < 0.05 was considered significant.

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Supplementary Figure 1. Postoperative venous remodeling and LOX localization in the rat femoral-epigastric AVF. A) Representative hematoxylin and eosin (H&E) stained venous sections of rat fistulas harvested at 5, 20, and 33 days post surgery. Notice that most wall remodeling has already occurred by the third week after surgery. B) Immunohistochemistry staining of LOX at day 20 after AVF creation.
Supplementary Figure 2. Postoperative gene expression (GE) of vascular remodeling related genes in the rat femoral-epigastric AVF. Fistulas were harvested at 5, 20, and 33 days post-surgery. Gene expression was normalized with respect to GAPDH and expressed as fold change vs. the contralateral vein at the time of AVF creation. Each point represents the mean ± SEM, n=3-6 per time point. *P<0.05, **P<0.01. Gene abbreviations: LOX, lysyl oxidase; MMP2 and MMP9, matrix metalloproteinases 2 and 9; ELN, elastin; FBLN5, fibulin 5; FN1, fibronectin; FGFR2, fibroblast growth factor receptor 2; COL1A1, COL3A1, and COL5A1, alpha 1 chains of collagens types I, III, and V; TIMP1, TIMP2, and TIMP4, TIMP metalloproteinase inhibitors 1, 2, and 4.
Supplementary Figure 3. Fibrosis and wall thickness in BAPN-treated and control AVFs. A) Representative Trichrome stained venous sections of rat AVFs harvested at 21 days post surgery. Animals were treated with systemic or local (via scaffold) BAPN or vehicle. Quantifications are presented in Figures 3C and 4D. Dashed boxes indicate areas magnified in Figure 3C. B) Average wall thickness in the four treatment groups. Data are presented as mean ± SEM (n=5-9 per group). **P<0.01, ***P<0.001
Supplementary Figure 4. Elastin content in BAPN-treated and control AVFs. A) Representative immunohistochemistry stainings of elastin in venous sections from rat AVFs harvested at 21 days post surgery. Animals were treated with systemic or local (via scaffold) BAPN or vehicle. B) Quantification of elastin content as percent of wall area in the four treatment groups. Data are presented as mean ± SEM (n=4-6 per group). *P<0.05
Supplementary Figure 5. Scanning electron microphotograph of BAPN-loaded PLGA nanofibers. Electrospinning results in a bulk, microporous scaffold comprised of individual nanofibers. Fibers were anisotropic with randomly oriented fibers. The darker, ballooning fibers are indicative of the presence of BAPN within the polymer matrix. Scale bar: 10 μm.
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MATERIALS AND METHODS

Human Veins and AVFs

Paraffin-embedded cross-sections of pre-access veins (N=20, 10 matured and 10 failed after AVF creation) from patients who received a planned two-stage upper arm AVF at Jackson Memorial Hospital or the University of Miami (UM) were randomly selected from the UM Vascular Biobank. In addition, 18 independent AVF specimens (8 matured and 10 failed) preserved in RNAlater (Qiagen) and collected at the time of second-stage surgery were randomly obtained from the biorepository. All AVFs were created by a single vascular surgeon (M.T.). Anatomic non-maturation was defined as an AVF that never achieved an internal diameter ≥ 6 mm. The time from access creation to second-stage surgery was similar between AVFs that matured and those that failed (74.4 ± 27.1 vs. 66.7 ± 28.5 days, respectively; P=0.57), and both subgroups were comparable in terms of demographics and comorbidities. The second-stage surgery consisted of a standard AVF transposition for AVFs that matured or a salvage procedure (short transposition, ligation, or AVG extension) for those that failed. Enrolled patients were consented before sample collection at the corresponding surgeries. The study was performed according to the ethical principles of the Declaration of Helsinki and regulatory requirements at both institutions. The ethics committee and Institutional Review Board at the University of Miami approved the study.

Rat model of AVF maturation

Sprague Dawley rats (280-320 grams) were purchased from Envigo (Indianapolis, IN). AVFs were created by an end-to-side anastomosis of the epigastric
vein to the nearby femoral artery (1). Ninety-six rats of both sexes were randomly allocated to experimental groups. Molecular analysis of AVFs was performed at 0, 5, 20, and 33 days post-surgery (n=18). For systemic drug administration, rats were given a daily intraperitoneal injection of BAPN (100 mg/kg; Sigma-Aldrich, St. Louis, MO) (n=21) or PBS (vehicle, n=21) on days -2 to 21 post surgery. For local delivery, scaffolds with BAPN (n=18) or PBS (n=18) were wrapped around the epigastric vein immediately after AVF creation. Flow was measured using a flowmeter (Transonic Systems Inc., Ithaca, NY) prior to AVF harvesting at day 21. Rats were euthanized with isoflurane, after which AVFs were removed and placed in ice-cold normal saline for pressure myography, RNAlater for qRT-PCR, formalin for histology, or liquid nitrogen for collagen crosslinking analyses. The overall attrition rate was 4.2%, including 3 animals with postsurgical complications and one animal excluded due to technical reasons (see below).

Two out of the 42 rats used for systemic BAPN/vehicle injections were excluded due to complications with surgical sutures. From the remaining 40, 11 were used for intraoperative flow measurements followed by myography (3 males [M] and 3 females [F] in the vehicle group, 3M and 2F in the BAPN arm), 18 independent animals were used to measure fibrosis (5M and 4F in both experimental arms), and 11 independent rats were used for mass spectrometry (3M and 3F in the vehicle group, 2M and 3F in the BAPN arm).

One rat from the 36 included in the scaffold experiments was euthanized due to excessive postoperative bleeding. From the remaining 35, 11 were used for intraoperative flow measurements (3M and 2F in the vehicle group, 3M and 3F in the BAPN arm). One additional AVF in the vehicle arm was damaged during flow evaluation
and had to be excluded from the subsequent myography experiments. The final set for myography consisted of 2M and 2F in the vehicle arm, and the remaining 3M and 3F in the BAPN group). Eleven independent rats were used to measure fibrosis (2M and 3F in the vehicle group, 3M and 3F in the BAPN arm), and 12 more for mass spectrometry (3M and 3F in both experimental arms).

We did not suffer any attrition in the group of 18 rats used for molecular analyses. The four time points when gene expression was measured (0, 5, 20, and 33 days) included similar numbers of male and female animals: 2M and 1F at time zero, 3M and 3F at 5d, 3M and 3F at 20d, and 2M and 1F at 33d.

**Scaffold fabrication and characterization**

Scaffolds were fabricated using an electrospinning apparatus under the direction of F.M.A. Ester-terminated PLGA (Resomer® RG 504) was purchased from Sigma-Aldrich. PLGA (15% w/v) and drug (10% w/w PLGA) were dissolved in hexafluoroisopropyl alcohol and stirred overnight. PLGA solutions with and without drug were loaded into a 3 mL syringe attached to a blunt 22G stainless steel needle. A 20-kV voltage was applied to the needle. Flow was maintained at 2.0 mL/h using an Aladdin Single-Syringe Pump (World Precision Instruments, Sarasota, FL). Nanofibers were deposited on an aluminum-wrapped collector located 13 cm from the needle tip. Scaffolds were dried overnight in a fume hood to remove any residual solvent. Fiber architecture was analyzed using scanning electron microscopy (SEM) (JEOL Ltd., Tokyo, Japan) after gold sputter-coating. Fiber diameter was determined in ImageJ
(National Institutes of Health, Bethesda, MD) by adding scale bars to the width of randomly selected fibers before averaging. For degradation, pre-weighed sections of PLGA scaffolds were placed in 1.5 mL of PBS and incubated in a water bath at 37°C. Scaffolds were then removed from the PBS, lyophilized, and weighed, before incubating in fresh PBS again until the next time point. The “old” PBS was stored for mass spectrometry (MS). Cumulative BAPN release was determined in tandem with degradation experiments. Percent release was calculated as the ratio of BAPN in the supernatant to the initial amount of BAPN in the PLGA scaffold.

**Mass spectrometry**

Cumulative BAPN release was quantified with a direct infusion/ triple quadrupole mass spectrometer (TSQ)(2) configured in positive mode, 42 eV collision energy, [10.000-150.000 m/z] scan range, and parent-ion scan mode. Stock solutions of BAPN (1 mg/mL) and aminoacetonitrile internal standards (IS; 1 mg/mL) were prepared in methanol to establish the Internal Response Factor (IRF), (Equation I). After calculating the IRF, the Amount Specific Compound (SC) of BAPN (m/z=71.42) was determined in relation to the IS (m/z=57.47) at different time points over 60 days (Equation II).

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\text{Internal Response Factor} = \frac{\text{Intensity}_{IS} \times \text{Amount}_{SC}}{\text{Amount}_{IS} \times \text{Intensity}_{SC}} \quad (I)
\]

\[
\text{Amount Specific Compound} = \frac{\text{Amount}_{IS} \times \text{Intensity}_{SC} \times \text{IRF}_{SC}}{\text{Intensity}_{IS}} \quad (II)
\]
Immature (hydroxylysinonorleucine [HLNL], dihydroxylysinonorleucine [DHLNL]) and mature crosslinks (pyridinoline [PYD], deoxypyridinoline [DPD]) were quantified as previously described.(3) Briefly, human and rat AVF tissues were lyophilized overnight, reduced with sodium borohydride (NaBH₄) in NaOH, and hydrolyzed overnight with 6N HCl. Samples were lyophilized again and injected (5 µL) into an Acclaim™ Polar Advantage II LC reverse-phase column (Thermo Scientific™). MS was performed with positive electrospray ionization (ESI) with parameters and MRM transitions optimized for immature and mature crosslinks detection.(3) Crosslinks were quantified using the ratio of peak area of epinephrine to pyridoxine as IS. The calibration curves for HLNL, DHLNL, PYD, DPD, and hydroxyproline were established through a linear least-squares regression with a weighing factor of 1/C². Immature and mature crosslinking density is expressed as the amounts of crosslinks per mg of dry weight of tissue.

Pressure myography

Rat AVFs were collected and cleaned of fat at 21 days post-surgery. The proximal and distal segment of the AVF were placed on a pair of steel cannulas (outer diameter: 900 or 1200 µm, depending on fistula size) of a pressure myograph system, DMT Model 110P (Danish Myo Technology, Ann Arbor, MI) and secured with surgical nylon sutures. After the distal segment of the artery was secured, and the vessel length adjusted to eliminate stretch, the intraluminal pressure was raised to 70 mmHg. The mounted vessels were equilibrated for 30 min at 37°C in calcium-free PSS gassed with a mixture of 95% O₂ and 5% CO₂, before reducing the intraluminal pressure to 3 mmHg. To obtain the pressure-diameter curve, the internal and external diameters were
measured while increasing intraluminal pressure in 10 mmHg steps between 3 and 130 mmHg. The diameters were recorded using the DMT MyoView 4 software (Danish Myo Technology). Distensibility was calculated as % deformation at increasing pressures with respect to the external diameter at 3 mmHg. The circumferential wall strain and stress and incremental elastic modulus (E_{inc}) were calculated as previously described.(4)

**Immunohistochemistry**

Paraffin-embedded rat AVFs were stained with Masson’s trichrome to quantify wall fibrosis. Percent area of fibrosis was calculated in ImageJ using color thresholding. Briefly, images were converted to RGB format and color thresholds on the blue channel were used to segment the blue (collagen) from the red/pink (cells) staining.

For staining of LOX in human and rat AVFs, cross-sections were rehydrated by serially immersing them in xylene, alcohol, and water. Antigen retrieval for LOX staining was performed by boiling slides in 10 mM citrate buffer (pH 6.0) for 20 minutes. Antigen retrieval for elastin was done using proteinase K digestion for 10 minutes at 37°C. Next, sections were blocked for 20 minutes with Tris–Borate saline supplemented with 15% fetal bovine serum (FBS), followed by 3% hydrogen peroxide. Veins and AVFs were incubated with either anti-LOX polyclonal antibody at room temperature for 1 hour (Cat# ab31238, 1:200, Abcam, Cambridge, UK) or anti-elastin polyclonal antibody (Cat# ab23748, 1:200, Abcam) overnight. Bound antibodies were detected using the Dako Universal Link kit (Agilent, Santa Clara, CA), and color was developed with the Dako DAB+ Substrate Chromogen System (Agilent). Nuclei were counterstained with Meyer's
hematoxylin and mounted in Entellan (Sigma-Aldrich). Images were acquired using a VisionTek DM01 digital microscope (Sakura Finetek, Torrance, CA). Operators blinded to the clinical data performed digital processing of images.

**Gene expression**

Gene expression of Lox and LoxL genes was measured in rat AVFs harvested 5 days after surgery. For ECM genes and remodeling factors, tissues were collected at days 5, 20, and 33 after surgery. Samples were immediately stored in RNALater at −80°C. Total RNA was isolated and reverse-transcribed as previously described.(5) Changes in gene expression were assessed using the following TaqMan Gene Expression Assays (Applied Biosystems, Waltham, MA): *Col1a1*, Rn01463848_m1; *Col3a1*, Rn01437681_m1; *Col5a1*, Rn00593170_m1; *Eln*, Rn01499782_m1; *Fbln5*, Rn00569712_m1; *Fgfr2*, Rn01269940_m1; *Fn1*, Rn00569575_m1; *Gapdh*, Rn99999916_s1; *Lox*, Rn01491829_m1; *Loxl1*, Rn01418038_m1; *Loxl2*, Rn01466080_m1; *Loxl3*, Rn01765241_m1; *Loxl4*, Rn01410872_m1; *Mmp2*, Rn01538170_m1; *Mmp9*, Rn00579162_m1; *Timp1*, Rn00587558_m1; *Timp2*, Rn00573232_m1; and *Timp4*, Rn01459160_m1. Real-time PCR was performed on an ABI Prism 7500 Fast Real-Time PCR System (96-well plate; Applied Biosystems). Relative gene expression was determined using the ΔΔCT method.(6) Gene expression was normalized with respect to GAPDH and expressed as fold change vs. the contralateral vein at the time of AVF creation.
Statistics

Statistical analyses were performed using GraphPad Prism version 8 for Windows (GraphPad Software, San Diego, CA). Normally distributed data were compared using unpaired t-tests with Welch’s correction and expressed as mean ± standard deviation (or mean ± standard error of the mean [SEM] where indicated in figures). Non-normally distributed data were compared using the Mann-Whitney test and expressed as median and interquartile range (IQR). A P value < 0.05 was considered significant.

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Supplementary Figure 1. Postoperative venous remodeling and LOX localization in the rat femoral-epigastric AVF. A) Representative hematoxylin and eosin (H&E) stained venous sections of rat fistulas harvested at 5, 20, and 33 days post surgery. Notice that most wall remodeling has already occurred by the third week after surgery. B) Immunohistochemistry staining of LOX at day 20 after AVF creation.
Supplementary Figure 2. Postoperative gene expression (GE) of vascular remodeling related genes in the rat femoral-epigastric AVF. Fistulas were harvested at 5, 20, and 33 days post-surgery. Gene expression was normalized with respect to GAPDH and expressed as fold change vs. the contralateral vein at the time of AVF creation. Each point represents the mean ± SEM, n=3-6 per time point. *P<0.05, **P<0.01. Gene abbreviations: LOX, lysyl oxidase; MMP2 and MMP9, matrix metalloproteinases 2 and 9; ELN, elastin; FBLN5, fibulin 5; FN1, fibronectin; FGFR2, fibroblast growth factor receptor 2; COL1A1, COL3A1, and COL5A1, alpha 1 chains of collagens types I, III, and V; TIMP1, TIMP2, and TIMP4, TIMP metalloproteinase inhibitors 1, 2, and 4.
Supplementary Figure 3. Fibrosis and wall thickness in BAPN-treated and control AVFs. A) Representative Trichrome stained venous sections of rat AVFs harvested at 21 days post surgery. Animals were treated with systemic or local (via scaffold) BAPN or vehicle. Quantifications are presented in Figures 3C and 4D. Dashed boxes indicate areas magnified in Figure 3C. B) Average wall thickness in the four treatment groups. Data are presented as mean ± SEM (n=5-9 per group). **P<0.01, ***P<0.001
Supplementary Figure 4. Elastin content in BAPN-treated and control AVFs. A) Representative immunohistochemistry stainings of elastin in venous sections from rat AVFs harvested at 21 days post surgery. Animals were treated with systemic or local (via scaffold) BAPN or vehicle. B) Quantification of elastin content as percent of wall area in the four treatment groups. Data are presented as mean ± SEM (n=4-6 per group). *P<0.05
Supplementary Figure 5. Scanning electron microphotograph of BAPN-loaded PLGA nanofibers. Electrospinning results in a bulk, microporous scaffold comprised of individual nanofibers. Fibers were anisotropic with randomly oriented fibers. The darker, ballooning fibers are indicative of the presence of BAPN within the polymer matrix. Scale bar: 10 μm.