Short-term pre-operative dietary restriction in vascular surgery

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Chapter 5.

Periprocedural Hydrogen Sulfide Therapy Improves Vascular Remodeling and Attenuates Vein Graft Disease.

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Subject terms: Vascular Disease, Cardiovascular Surgery, Diet and Nutrition

Abstract.

Background: Failure rates after revascularization surgery remain high, both in vein grafts (VG) and arterial interventions. One promising approach to improve outcomes is endogenous upregulation of the gaseous transmitter-molecule hydrogen sulfide (H_2S), via short-term dietary restriction. However, strict patient-compliance stands as a potential translational barrier in the vascular surgery patient-population. Here we present a new therapeutic approach, via a locally applicable gel containing the H_2S-donor GYY, to both mitigate graft failure and improve arterial remodeling.

Methods and Results: All experiments were performed on C57BL/6 (male, 12-weeks old) mice. VG surgery was performed by grafting a donor-mouse cava vein into the right common carotid artery (RCCA) of a recipient via an end-to-end anastomosis. In separate experiments arterial intimal hyperplasia was assayed via a RCCA focal stenosis model. All mice were harvested at post-op day-28 and artery/graft was processed for histology. Efficacy of H_2S was first tested via GYY-supplementation of drinking water either 1-week before VG surgery (Pre-GYY) or starting immediately postoperatively (Post-GYY).

Pre-GYY mice had a 36.5% decrease in intimal/media+adventitia area ratio compared to controls. GYY in a 40% Pluronic-gel (or vehicle) locally applied to the graft/artery had decreased intimal/media area ratios (RCCA) and improved vessel diameters. GYY-gel treated VG had larger diameters at both POD14 and POD28, and a 56.7% reduction in intimal/media+adventitia area ratios. Intimal VSMC migration was decreased 30.6% after GYY-gel treatment, which was reproduced *in-vitro*.

Conclusion(s): Local gel-based treatment with the H_2S-donor GYY stands as a translatable therapeutic to improve VG durability and arterial remodeling after injury.

1. Introduction.

In lower extremity bypass surgery, autologous vein grafts remain the superior choice to achieve successful revascularization.[1] Although success rates are surpassing other conduits,[2] vein graft primary patency nevertheless falls towards 60% one-year post-intervention[3] with vein graft disease (VGD) as the main effector in prompting graft occlusion mid to long-term post-implantation.[4] The intimal hyperplasia (IH) seen in VGD, i.e. thickening of the intimal wall by vascular smooth muscle cell (VSMC) growth[5], also drives open and endovascular arterial intervention failure. Taken together both these components render the vascular fibroproliferative response to injury a major unaddressed clinical challenge in cardiovascular surgery practice.

An emerging scientific field holding translational promise employs the concept of preconditioning of the patient, prior to surgery, against surgical stress/injury via short-term dietary restriction (DR). The efficacy of DR, including the restriction of calories, proteins or specific amino acids, is established in a wide range of pre-clinical surgical models, including surgical trauma,[6, 7] renal[8, 9] and hepatic[8, 10, 11] ischemia-reperfusion injury, focal stroke in the brain,[12] post-stenosis arterial hyperplasia[13] and hindlimb ischemia,[14] without decelerating post-interventional wound healing processes.[15]

One mechanism by which DR derives its benefit, is via increased production of endogenous hydrogen sulfide (H_2S)[16], a gaseous vasodilator and transmitter molecule[17] with anti-inflammatory and cytoprotective potential.[18] In the vasculature, H_2S is mainly enzymatically derived, with cystathionine *γ*-lyase (CGL) being the most abundant H_2S producing enzyme.[19] In the setting of vascular injury in rodent models, DR-induced augmentation of endogenous H_2S protects from ischemia-reperfusion injury[20] and accelerates neovascularization after hindlimb ischemia.[14] Genetic (cardiac-specific) overexpression of CGL protects from heart failure after transverse aortic constriction[21], while knockdown of the same gene leads to increased neointima formation after carotid ligation, via increased VSMC migration.[22]

In venous bypass surgery, DR-induced increased endogenous H_2S production (via CGL upregulation) attenuates inward remodeling and improves vein graft adaptation to its arterial environment, partly via limiting both VSMC migration and neutrophil transmigration towards the intimal layer.[23] Upregulation of endogenous H_2S therefore possesses the capability to improve outcomes in

various settings of (vascular) surgical injury. Nonetheless, translational barriers remain, not least of which includes the requirement for pre-planning of the dietary intervention, as we found in a recent pilot study of DR in patients scheduled for vascular surgery.[24]

Thus making DR-mimetic drugs, including direct H_2S administration, an attractive alternative approach. Although systemic treatment with various H_2S donors shows therapeutic potential in a wide range of preclinical disease models[25], including a single dose immediately prior to hepatic ischemia reperfusion injury[16], its potential in vascular reconstructions has yet to be fully explored. Furthermore, the ability to use local delivery may address potential safety issues regarding systemic treatments, thereby improving clinical translatability. Here, we tested the potential of H_2S to mitigate IH and VGD, using a single, local periprocedural application.

2. Materials & Methods.

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

2.1 Experimental Animals.

All animal experiments were approved by the appropriate Harvard Medical Area or Brigham and Women's Hospital Institutional Animal Care and Use Committee (04475 and A4752-01 respectively) and in accordance with the NIH guidelines. All surgical experiments were performed on C57BL/6 mice (male, 10-12 weeks old, Stock No: 000664, Jackson Laboratory), fed a high-fat diet (Research Diets D12492, 60% fat) during the experiment. Mice were housed 4-5 per cage and maintained on a 12-hour light-dark cycle at 22°C with 30-50% humidity. All *in-vivo* experiments were conducted in male mice to limit inter-group variability while assessing intervention effectiveness, thereby lowering the number of experimental animals needed.

2.2 Vein Graft Surgery.

Vein graft surgery was performed as described previously.[26] Briefly, mice were anesthetized with 5% isoflurane and maintained under 2-3% isoflurane via a nose cone for the duration of the procedure. The mouse was placed on a heating pad to maintain periprocedural body temperature. After removal of fur in the neck region a neckline incision was performed. After dissection of the right common carotid artery (RCCA) from its surrounding tissues, the artery was ligated with an 8-0 nylon suture. Vascular clamps were placed at the proximal and distal arterial ends and the carotid wall was everted over a polyetheretherketone cuff. The thoracic inferior caval vein from a donor mouse was harvested just before the start of the vein graft procedure in the recipient and stored in ice cold sterile 0.9% NaCl supplemented with heparin (100UI/mL). After everting the carotid artery of the recipient over the cuff, the donor caval vein was sleeved and then sutured over both ends of the cuff with an 8-0 nylon suture, creating an end-to-end anastomosis. After grafting of the caval vein, vascular clamps were removed, and blood flow was restored. The incision was closed with 6-0 Vicryl sutures. Post-operatively animals received warm lactate ringer solution (0.5mL, subcutaneous) and buprenorphine (0.1mg/kg, subcutaneous).

2.3 Focal Carotid Stenosis Procedure.

A focal stenosis was created as described previously to generate a hemodynamically induced arterial intimal hyperplastic response in the setting of flowing blood.[27] Briefly, mice were anesthetized with 5% isoflurane and maintained under 2-3% isoflurane via nose cone for the duration of the procedure. After dissection of the RCCA from its surrounding tissues, a 35-gauge blunt needle mandrel was placed longitudinally along the RCCA and tied with a 9-0 nylon suture approximately 2-2.5mm proximal to the bifurcation. The needle mandrel was then removed and skin closed with a 6-0 Vicryl suture. Post-operatively mice received warm lactate ringer solution (0.5mL, subcutaneous) and buprenorphine (0.1mg/kg, subcutaneous).

2.4 Systemic Treatment with GYY4137.

For systemic treatment, mice were randomized in Pre-GYY, Post-GYY and control groups. Based on our previous work[16], in the Pre-GYY treatment group, 250μM GYY4137 (Sigma, cat# SML0100) (GYY) was supplied in the drinking water for one-week preceding vein graft surgery and replaced fresh after 3 days. Immediately post-surgery, drinking water was replaced with regular (without GYY). In the Post-GYY treatment group, 250 μM GYY was supplemented in the drinking water immediately post-operative until harvest at post-op day 28 and replaced weekly. [28]

2.5 Periprocedural Local Treatment with GYY.

For periprocedural local treatment with GYY, a 40% Pluronic gel (Sigma, cat# P2443) was created the day before surgery (40% w/v, 4-gram Pluronic gel, 10 mL sterile 0.9% NaCl) and kept at 4°C overnight under continuous stirring. Mice were randomized into GYY-treatment or vehicle groups. Ten minutes before application of the gel onto the vessel/graft, GYY was dissolved into a stock solution (0.9% NaCl) and resuspended in 100µL of 40% Pluronic gel to a final concentration of 250µM. During the focal stenosis procedure, 100 µL gel containing GYY or vehicle only was applied immediately after stenosis creation. During bypass surgery, the gel (GYY or vehicle) was applied onto the caval vein after it was grafted into the RCCA but before opening the proximal and distal vascular clamps. Immediately after enveloping the graft with the gel, vascular clamps were opened, and blood flow was restored.

2.6 Vein Graft/Focal Carotid Stenosis Harvest.

Animals were anesthetized (induction under 5% isoflurane, 1-2% continued isoflurane to maintain anesthesia), anesthetic depth was confirmed by toe pinch. A percutaneous cardiac puncture was performed to obtain whole blood, followed by a thoracotomy. Mice were then euthanized via exsanguination by cutting and removing the caval vein. Perfusion was performed with lactate Ringers solution via the left ventricle for 3 minutes, then switched to 3 minutes of perfusion-fixation with 10% formalin. To harvest the graft/RCCA, a midline neck incision was made, the graft/RCCA was excised en-block and fixed in 10% formalin for 24 hours. After 24 hours the tissue was transferred to a 70% ethanol solution and then processed for paraffin embedding.

2.7 Graft & Artery Processing for Histology.

After paraffin embedding, both vein grafts and RCCA were cut with a microtome in 5µm sections and mounted on slides. Grafts were sectioned at regular intervals of 200 µm, starting from the proximal cuff till 1000µm post proximal cuff. Arteries were cut at regular intervals of 200µm, starting at 200µm proximal from the focal stenosis, till 2800µm proximal from the stenosis. After section cutting, a Masson-trichome histology staining was performed. In short, slides were deparaffinized to 95% ethanol, 3 minutes in 5% picric acid (in 95% ethanol), tap water wash, 3 minute stain in working Harris Hematoxylin Solution (Fisher 213 Scientific, cat# 245-678), tap water wash, stained with 1% Biebrich Scarlet in 1% acetic acid (Fisher Scientific, cat# A38S-500) for 3 minutes, quick rinse in distilled water, 1 minute stain in 5% Phosphomolybdic/Phosphotungstic acid solution then stained with 2.5% light green SF yellowish in 2.5% acetic acid (Fisher Scientific, cat# A38S-500) for 4 minutes, rinsed in distilled water and rinsed in 1% acetic acid solution (Fisher Scientific, cat# A38S-500) for 2 minutes. After dehydration with Xylene slides were covered with a cover glass employing Permount (Electron Microscopy Science, cat# 17986-05).

2.8 Immunohistochemistry.

For fluorescent immunohistochemistry (IHC), slides were first pre-heated to 60°C in a vacuum oven for 30 minutes. Afterwards, slides were immediately deparaffinized (Xylene 3x5 minutes, 100% ethanol 3x5 minutes, 70% ethanol 5 minutes, 50% ethanol 5 minutes, deionized water 5 minutes). After deparaffinization, antigen retrieval was performed for 30 minutes at 97°C in citrate buffer (pH 6.0 in PBS, Abcam, ab93678) and slides were cooled to room temperature (RT) afterwards. Next, slides were incubated with 10% goat serum (Life Technologies, cat#50062Z), in PBS with 0.3M glycine (Aijnomoto, #R015N0080039), for 1 hour at RT. Consecutively, slides were incubated with primary antibodies for SMC-α (mouse anti-mouse, Abcam, ab7817, 1:800 dilution) and Ki-67 (Ki-67 (rabbit anti-mouse, Abcam, ab16667, 1:100 dilution) o/n at 4°C for SMC-α – Ki-67 double staining. The next day, slides were washed 3 times in PBS + 0.05% tween and incubated in secondary antibody for 2 hours at RT. For SMC-α + Ki-67 double staining, slides were incubated with Alexa Fluor 647 (goat anti-mouse, A-32728, 1:600 dilution) and Alexa Flour 568 (goat anti-rabbit, A-11011, 1:600 dilution). After secondary antibody incubation, slides were washed in PBS + 0.05% tween 3x5 minutes and mounted with DAPI (Vector, CB-1000) and imaged by confocal microscopy.

2.9 Histology and Immunohistochemistry Analyses.

All histology and immunohistochemistry analysis were done by a blinded observer. Vein grafts were excluded from analysis in case of complete occlusion of the graft/RCCA at POD28. Based on our previous vein graft experiments with endogenous H_2S treatment[23], our initial systemic GYY experiments were conducted with n=10/group. After systemic GYY experiments (n=10/group), 2 control, 2 Pre-GYY and 2 Post-GYY vein grafts were occluded at POD28. Observed differences between experimental groups after systemic GYY treatment led us to increase group size from n=10 to n=18 for local (one-time) treatment with GYY. For local GYY experiments (n=18/group), 0 vehicle and 2 local-GYY vein grafts were occluded at POD28. For focal stenosis experiments(n=10/group), 1 vehicle and 0 local-GYY RCCA were occluded at POD28. Failure rates after both vein graft and focal stenosis experiments were comparable to previously conducted experiments by microsurgeon (~10%).

Brightfield images of vein graft and carotid artery cross-sections were taken with a Zeiss Axio A1 microscope (Carl Zeiss). For vein grafts, histomorphometric analysis was performed on 5 cross sections per vein graft (200-400-600-800-1000μm) using Image J 1.51p (Java 1.8.0_66).

Luminal, intimal (I), medial and adventitial (M+A) areas and circumferences were measured for each cross section with 3 cross sections per slide, then averaged per slide. Next, I/M+A area, intimal thickness, M+A thickness, I/M+A thickness ratios and corrected luminal area were calculated as described previously,[29] to account for morphometric changes induced by vein graft processing. For collagen measurements, Masson-trichome stained slides were processed via the color deconvolution tool in Image J and percentage of total vein graft layer occupied by the deconvoluted green channel was then calculated. For VSMC + Ki-67 analysis, images were processed in Image J 1.51p by measuring intimal and M+A total area based on DAPI positive cells, followed by color thresholding of the SMC-α positive cells. Area occupied by SMC-α was then calculated as a percentage of total area in that respective vein graft layer. Proliferating VSMCs were defined was SMC-α / Ki-67 double-positive cells, counted per vein graft layer and normalized to mm^2. For histomorphometric analysis of carotid artery cross sections, luminal, I and M circumferences and area were measured. Followed by calculation of I/M area, M thickness, I/M thickness ratio and corrected luminal diameter.

2.10 Duplex Ultrasound Biomicroscopy.

A Vevo 2100 imaging system with 18-70-MhZ linear array transducers (VisualSonics Inc., Toronto, ON, Canada) was employed for high resolution *in vivo* ultrasonography of vein grafts and carotid arteries post-stenosis creation. At post-operative day 14 and 28 mice were anesthetized via 5% isoflurane inhalation and maintained under 2-3% via nose cone inhalation, body temperature was controlled via heating pad. To measure vessel cross-dimensional sections, M-mode was employed. Three luminal axial images were taken (at proximal, mid and distal graft/post-stenosis) and mean vessel diameters were calculated.

2.11 Primary Vascular Smooth Muscle Cell Isolation.

Primary VSMCs were harvested from murine aortas as described before.[30] Briefly, C57BL/6 (4-6 weeks old) mice were anesthetized (induction under 5% isoflurane, 1-2% continues isoflurane to maintain anesthesia) and a thoracoabdominal incision was performed after which the thoracic aorta was exposed. Primary euthanasia occurred via removal of the cava vein followed by exsanguination. The left cardiac ventricle was perfused with sterile saline and the perivascular adipose tissue surrounding the aorta was carefully removed with forceps and scissors. The thoracic aorta was removed and placed in Hanks Balanced Salt Solution [HBSS] (Thermo Fisher Scientific, cat#14025076) with 1% penicillin/streptomycin (Corning, cat#30-002-CL) on ice. After a brief wash in PBS, aortas where then placed in 6-well plates (n=2 per well) in enzyme digestion solution consisting of 1 mg/mL Collagenase type 2 (Gibco, cat#17101015), 0.24 mg/mL Elastase (Worthington Biochemical, LS002279), 1mg/mL Soybean Trypsin Inhibitor (Worthington Biochemical, LS0033570), 1% penicillin/streptomycin in HBSS at 37°C/5% CO_2 for 10 min. After the first digestion the aortas were transferred to another 6-well well containing Dulbecco's Modified Eagle Medium (DMEM) (Thermo Fisher Scientific, cat#10566016), 20% CCS, 1% pen/strep and washed briefly. Next, employing forceps, the adventitia layer of the aorta was carefully everted over the intimal and medial layer of the aorta and removed. After removal of the adventitial layer, the aorta was cut longitudinally, and the endothelium carefully scraped off with angled forceps. The artery was then placed in enzyme digestion solution and incubated for 60 minutes at 37°C/5% CO_2. After the second digestion, the resulting single cell suspension was washed with DMEM (20% CCS, 1% pen/strep), centrifuged for 5 minutes at 1200 r.p.m/4°C, resuspended in complete DMEM and plated in 24-well plates (Falcon, cat#353047). Cells were passaged at 90% confluency and used for subsequent assays at passage 3-8.

2.12 Human Umbilical Endothelial Cell (HUVEC) Isolation and Culture.

Cords were collected from full term pregnancies and stored in sterile PBS at 4°C and subsequently used within 7 days. A cannula was inserted in the vein and flushed with sterile PBS. The vein was infused with 0.075% collagenase type II (Worthington, Lakewood, NJ, USA) and incubated for 20 minutes at 37°C. The collagenase solution was collected, and the vein was flushed with PBS. The cell suspension was centrifuged at 1200 rpm for 5 minutes and the pellet was resuspended in complete culture medium, EGMTM-2 Endothelial Cell Growth Medium-2 (BulletKit, Lonza). The cells were cultured in plates coated with fibronectin from bovine plasma (Sigma).

Cells were maintained at 37°C in a humidified 5% CO2 environment. Culture medium was refreshed every 2-3 days. Cells were grown until cobblestone morphology was reached. Cells were passed 1:3 using trypsin-EDTA (Sigma) and HUVECs were used up to passage three.

2.13 MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay.

HUVEC and murine VSMCs, both p3-4, were seeded in gelatin coated 96-well plates (0.1% gelatin, Sigma, G9391) in complete medium and left to adhere overnight. 125-250-500-1000μM solutions of GYY4137 were prepared fresh in complete medium and supplemented in 6 replicate wells per cell type. After 2, 6 and 12 hours of incubation with GYY, 10μL of MTT (5mg/mL) was added per well and left to incubate for another 4 hours. After 4 hours of MTT incubation, supernatant was removed and replaced with 75μL of isopropanol/HCL and incubated for 90'. 96-well plates were then read at an optical density of 590nm with a plate reader.

2.14 Live-cell Imaging & Analysis.

HUVEC and murine VSMCs, both p3-4, were seeded in gelatin coated, tissue-cell treated 96-well plates in complete medium and left to adhere overnight. 125-250-500-1000 μM solutions of GYY4137 were prepared fresh in complete medium. For both VSMCs and HUVECs, propidium iodide (PI) (1:200) was added to the GYY containing medium and supplemented in duplicate wells. Live imaging was performed using a fluorescence microscope with an automated stage and temperature and CO_2 control (Leica AF6000) taking pictures every 15 minutes at two locations / well. The number of cells positive for PI was then counted per image and totaled per well.

2.15 *In- vitro* Transmigration Assay.

VSMCs were serum starved overnight (0.5% CCS) and seeded in the apical chamber of 6.5mm Transwell inserts with 8μm pore polyester membranes (Corning, cat#3464). Before seeding $5x10^4$ cells in DMEM per apical chamber, apical membranes were coated with collagen type 1 (Thermo Fisher Scientific, A1048301). VSMCs were seeded with or in absence of 250μM GYY. In a separate experiment, VSMCs were pre-incubated with 250μM GYY for 6hrs before seeding. The bottom chamber contained serum-free DMEM with/without 10ng/mL Platelet Derived Growth Factor-BB (PDGF-BB, R&D systems, cat#520-BB-050). After 6 hours incubation, apical and bottom chambers were washed with PBS twice and fixed in 4% paraformaldehyde (PFA, Chemcruz, cat# sc-281692) for 10 minutes at RT. Next, inserts were washed twice in PBS and incubated with DAPI for 10 minutes at RT in the dark. After washing with PBS, membranes were cut out from inserts and mounted on slides. Per insert, 8 images were taken at 20x magnification, number of migrated VSMCs were then counted with ImageJ and normalized per mm^2.

2.13 Immunocytochemistry.

VSMCs were first grown on gelatin-coated slides (0.1% gelatin, Sigma, G9391) till near-confluency then fixed with 4% PFA, washed twice in PBS and permeabilized with 0.01% Triton and incubated with 0.1% bovine serum albumin in PBS for 45 minutes. After removal of the blocking reagent, slides where incubated with SMC-α primary antibody (mouse anti-mouse, Abcam, ab7817, 1:400 dilution in 0.1% BSA/PBS) o/n at 4°C. After 3 washes with PBS, slides were then stained with Alexa Fluor 647 (goat anti-mouse, A-32728, 1:600 dilution in BSA/PBS) for 2 hours at RT. After 2 washes with PBS, slides were incubated with DAPI mounting medium and covered with coverslips. Slides were imaged by confocal microscope.

2.16 *Ex-vivo* measurement of H_2S.

To establish the release profile of GYY/NaHS *ex-vivo*, GYY (250μM, 1mM) and NaHS (1mM) were dissolved in 3mL PBS and incubated at 37°C. At various time-points, solutions were sampled and incubated with 0.25μM SF_7-AM (Tocris, cat#4943) for 30 minutes at 37°C in the dark in a glass bottom 96-well plate (Cellvis, cat#P96-0-N). After incubation, plate was imaged with a GE Typhoon FLA 9500 Laser Scanner (GE Healthcare, cat#15342) and mean fluorescent intensity of each well was measured with ImageJ. *H_2S release by GYY in a 40% Pluronic gel.* To assess the release profile of GYY while dissolved in a 40% Pluronic gel. PBS, GYY (1mM, 250μM) and NaHS (1mM, Sigma, cat#161527) were dissolved in 3mL 40% Pluronic gel in separate wells of a 6-well plate, and incubated at 37°C. After gel was solidified, each gel was overlaid with 3mL of sterile PBS. At various time-points, supernatant was sampled and incubated with 0.25μM SF_7-AM for 30 minutes at 37°C in the dark in a glass 96-well plate and imaged with a GE Typhoon laser scanner. Starting at 12 hours after first overlaying the Pluronic gel with PBS, supernatant was removed every 24 hours and renewed with fresh 3mL PBS.

H_2S release after local GYY treatment. C57BL/6 mice (n=4/group) were anesthetized via 5% isoflurane induction then switched to 2-3% isoflurane. Midline neck incision was performed and the right and left CCA were exposed

via surgical manipulation. Both arteries were then enveloped with the same Pluronic gel with or without 250μM GYY. After 30 seconds incubation, midline incision was closed with 6/0 Vicryl suture and post-operatively mice received warm lactate ringer solution (0.5mL, subcutaneous) and buprenorphine (0.1mg/kg, subcutaneous). 24 hours after surgery, mice were anesthetized, and the wound bed was re-opened. 100μL of gel remnant visible in the wound bed was collected and incubated with 0.25μM SF_7-AM for 30 minutes in a 96-well plate. Mice were then euthanized via cervical dislocation After incubation, plates were imaged with the GE Typhoon laser scanner, wells were then analyzed with ImageJ and corrected for background signal (PBS + 0.25μM SF_7-AM).

2.17 Statistical analyses.

Data are expressed as mean ± standard deviation (Mean ± SD). Normality testing was performed with a Shapiro-Wilk normality test. Student's t-test, one-way ANOVA or two-way ANOVA was used to analyze normally distributed data. Non-normally distributed data was analyzed by Mann-Whitney test or Kruskal-Wallis test. All statistical analyses were performed with Graphpad (8.12).

3. Results.

3.1 Systemic therapy with the H_2S donor GYY limits vein graft disease.

To test the effectiveness of exogenous H_2S in vein graft surgery, we first supplemented 250μM GYY to the drinking water of C57BL/6 mice, either throughout the 7 days preceding vein graft surgery (Pre-GYY) or starting immediately after surgery and continuing until harvest at POD28 (Post-GYY). **Fig. 1A** exemplifies the experimental design and **Fig. 1B** outlines the vein graft procedure, which encompasses the transplantation of a donor caval vein in a recipient RCCA via an end-to-end anastomosis. At POD28, all mice were anesthetized and then euthanized via exsanguination, vein grafts were harvested and processed for histology. **Fig. 1C** shows representative histology images of vein graft cross-sections from control, pre-GYY and post-GYY groups after Masson-trichome staining at POD28.

Pre-treatment with GYY decreased the I/M+A area and thickness ratios with 36.5% and 35.8% respectively (**Fig. 1D-E**) compared to control mice, while there was no difference in control versus post-GYY area and thickness ratios (**Fig. 1D-E**). In concurrence with a decrease in area and thickness ratios, intimal area (**Fig. 1F**) and thickness (**Fig. 1G**) were diminished in the pre-GYY group. While there was a trend towards decreased intimal area and thickness in the post-GYY group (**Fig. 1F-G**). In both treatment groups, there was no difference in lumen area (**Fig. S1A**), M+A area (**Fig. S1B)** and thickness (**Fig. S1C**). Further analysis of the VSMC content of pre- and post-conditioned vein grafts (**Fig. 1H**) hinted towards decreased intimal VSMC infiltration in the pre-GYY group (**Fig. 1H-I**). Interestingly, in the post-GYY group there was increased M+A collagen content (**Fig. S1D**) and a trend towards increased overall collagen content, signifying an outward remodeling ~~arterialization~~ response.

Taken together these data suggests a significant beneficial effect for systemic GYY treatment in attenuating VGD with pre-conditioning yielding an additional beneficial response compared to post-conditioning.

3.2 Development of a locally applicable Pluronic gel that ensures extended H_2S release both *ex-vivo* and *in-vivo*.

Due to potential translational barriers associated with preconditioning for extended time periods prior to surgery, we next explored the potential local use of GYY at the time of surgery to prevent potentially dangerous side effects associated with excess H_2S. To understand the kinetics of local delivery of different sulfide donors we first tested the slow and extended release qualities of different sulfide donors *ex-vivo* by dissolving the well-known sulfide donor sodium hydrosulfide (NaHS) as a positive control or GYY in PBS at 1mM and 1mM/250μM respectively.[28]

To first compare the presence of free H_2S at various time-points after dissolving slow- and fast-releasing H_2S-donors, we dissolved GYY and NaHS in PBS and took a 100μL sample at regular intervals. This sample was placed in a 96-well plate and free H_2S was then measured by adding the H_2S-binding molecular probe SF_7-AM.[31] As expected, PBS-dissolved GYY proved capable of extended release of H_2S molecules up until 1-day after dissolvement, while NaHS-derived H_2S release diminished after 6 hours (**Fig. 2A**). After the 24-hour time-point there was no GYY/PBS present due to evaporation. In the same experiment, we compared the sulfide release-rate of both donors, by a repeat-measurement of the T=0 sample, after SF_7-AM was added. We found that GYY maintained a steady and continuous release of H_2S molecules, as shown by a slow increase in intensity of the SF_7-AM signal. While the H_2S molecules released by NaHS rapidly saturated the SF_7-AM signal (**Fig. 2B**).

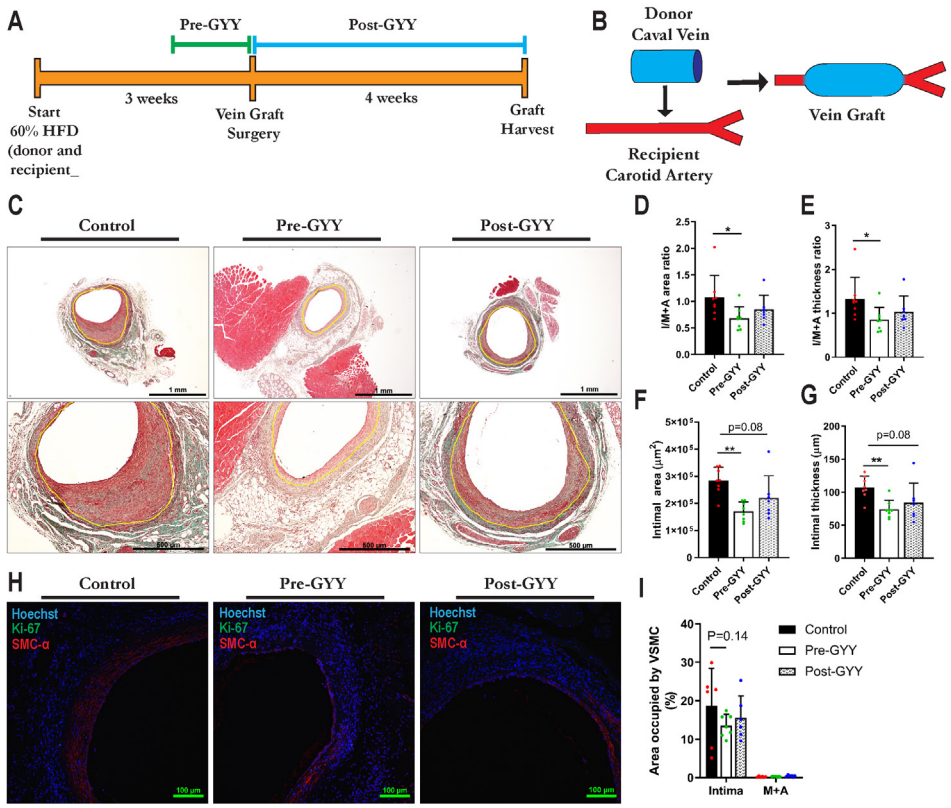


Figure 1. Systemic therapy with the H_2S donor GYY limits vein graft disease. **A:** Schematic depiction of the vein graft procedure: end-to-end anastomosis of a donor mouse caval vein into a recipient mouse right common carotid artery. **B:** Experimental design: 60% high-fat diet (HFD), 1 week of pre-conditioning (Pre-GYY) or 4 weeks of post-conditioning (Post-GYY) vs Control. **C:** Representative images of Control, Pre-GYY and Post-GYY vein grafts harvested at POD28 after Masson-trichome staining, with yellow lining highlighting the intimal-M+A border. Scale bars= 1mm or 500μm as indicated. **D-G**: Morphometric analysis of vein grafts at POD28 in Control, Pre-GYY and Post-GYY treated mice as indicated; n=7-8/group. Multiple comparisons are via Kruskal-Wallis test with Dunn's multiple comparisons test unless indicated otherwise. **D:** I/M+A area ratios. **E**: I/M+A thickness. **F**: Intimal area. **G**: Intimal thickness. **F-G:** One-way ANOVA with Dunnett's multiple comparisons test. **H:** Vein grafts after VSMC+Ki-67 IHC. Scale bars=100μm as indicated. **I**: Percent of intimal and M+A layers occupied by VSMCs via two-way ANOVA with Turkey's multiple comparisons test. *Data represented as: Mean ± SD. * p<0.05, **p<0.01*

Next, to enable periprocedural local sustained and targeted release, we tested whether dissolving GYY in a 40% Pluronic gel would extend the release profile of GYY. We dissolved PBS, GYY or NaHS in 40% Pluronic gel at 37°C, then overlaid the gel with PBS. At regular intervals, the supernatant was tested for free H_2S by incubation with the SF_7-AM probe. Measurements were continued until the Pluronic gel was dissolved in supernatant PBS and consecutively evaporated. Interestingly, we were able to extend the release-profile of GYY from 24 to 72 hours by utilizing Pluronic gel as a vehicle, while NaHS was fully released from the gel at 6 hours post-dissolvement (**Fig. 2C**).

Finally, we evaluated the capability of this GYY-gel to release H_2S *in vivo* by enveloping the left and right common carotid arteries (LCCA/RCCA) in the 40% Pluronic gel with/without 250μM GYY. At 24 hours post-surgery, we tested for H_2S release from the gel-remnants surrounding the LCCA/RCCA. Although sampling was hampered by high-back ground signal due to the presence of blood, there was a trend for an increase in fluorescent intensity after incubation with SF_7-AM in the local-GYY group (1±0.37 vs 1.791±0.72, n=4/group, p=0.1) supporting extended H_2S release *in-vivo*.

3.3 Local application of the H_2S donor GYY attenuates the arterial fibroproliferative response to injury.

To determine the effectiveness of local therapy with GYY during a vascular procedure, we first tested our GYY-gel in a model of arterial hyperplasia and vascular remodeling. **Fig. 3A** illustrates the employed surgical model of focal stenosis of the RCCA in order to create proximal arterial hyperplasia and remodeling, while **Fig 3B** outlines the experimental design.

Interestingly, at POD14 there was an increase in RCCA diameter proximal from the stenosis in the GYY group, as measured by ultrasound (**Fig. 3C**). After harvesting the RCCA at POD28, a Masson-trichome staining was performed with representative images in **Fig. 3D** at 200μm and 400μm proximal from the stenosis. **Fig. 3E** and **Fig. 3F** visualize the remodeling response at regular distance intervals pre-stenosis, per I/M area ratio and intimal area respectively. When averaged, the group treated with local GYY showed decreased I/M area and thickness (**Fig. 3G-H**) ratios, and decreased intimal thickness (**Fig. 3J**), while there was a trend for decreased intimal area (**Fig. 3I**). Local treatment with GYY had no significant effect on lumen area (**Fig. S2A)**, medial area (**Fig. S2B**) or medial thickness (**Fig. S2C**).

Thus, local therapy was effective in mitigating the remodeling response to vascular surgical injury.

3.4 Periprocedural H_2S therapy protects from vein graft disease and attenuates intimal VSMC migration.

Consequently, we set out to test whether this intervention could be advantageous in attenuating VGD and intimal hyperplasia. As outlined in **Fig. 4A**, we next applied our GYY-gel locally during the bypass procedure. During the procedure, the vein graft was enveloped with Pluronic gel (vehicle or GYY), just before the vascular clamps were opened. Interestingly, at POD14 and POD28, vein grafts treated with GYY showed a sustained increase in vein graft diameters *in-vivo* compared to vehicle treated grafts, as measured by ultrasound (**Fig. 4B**).

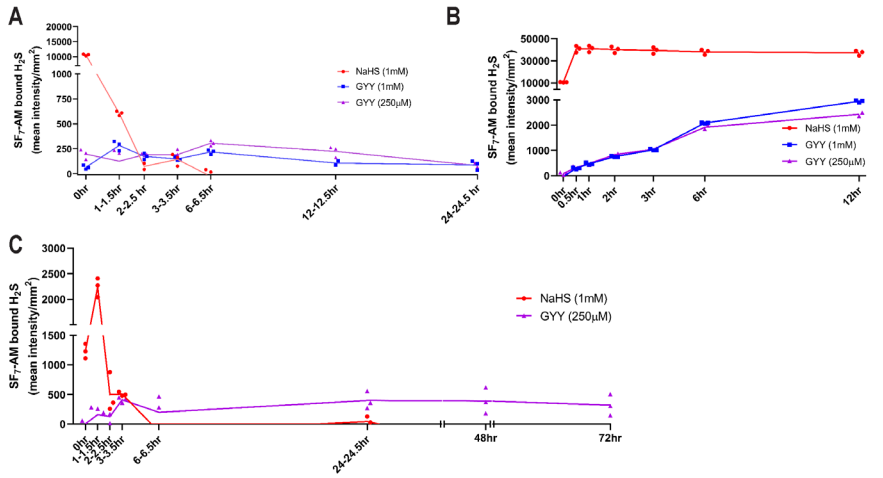


Figure 2. Development of a locally applicable pluronic gel that ensures extended H_2S release both ex-vivo and in-vivo

A-C: All samples were incubated with 0.25μM SF_7-AM in a 96-well plate in triplicates for 30 minutes at 37°C and then imaged. Resulting fluorescent intensity was corrected for background signal with a PBS + SF_7-AM control. **A:** NaHS (1mM), GYY (1mM, 250μM) and PBS dissolved in equal volume PBS, H_2S release was measured at regular intervals. **B:** Repeated measurement of same wells in 96-wells plate to assess release-rate of respective H_2S releasing compounds. **C**: H_2S-drugs dissolved in equal volume of 40% pluronic gel and overlain with equal volume PBS. At regular intervals H_2S release in PBS supernatant was measured.

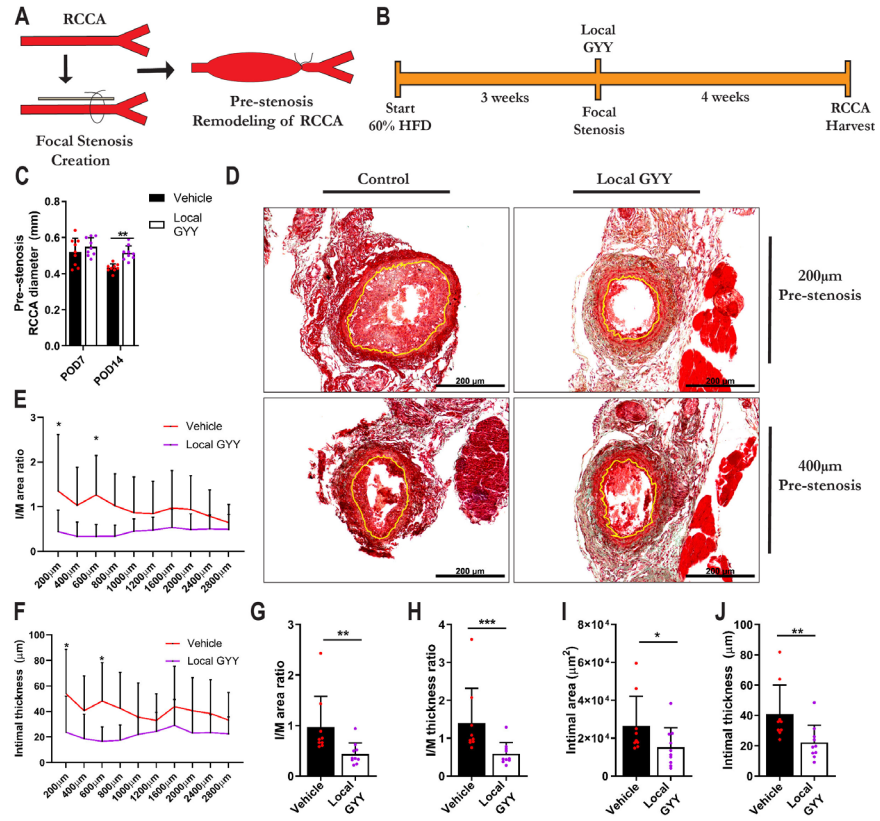


Figure 3. Local application of the H_2S donor GYY mitigates injury induced arterial intimal hyperplasia.

A: Schematic depiction of surgical procedure, with partial ligation of the right common carotid artery (RCCA) and resulting remodeling proximal from the stenosis. **B:** Experimental outline. **C:** Pre-stenosis diameter of RCCA at POD7 and POD14 in vehicle and local GYY treated animals, via two-way ANOVA, n=9-10/group. **D**: Masson-Trichome staining of RCCA cross-sections at POD28 after focal stenosis, with yellow lining indicating the intima-media border. Scale bars are 200μm as indicated. **E-F**: Measurement of I/M area and thickness ratio respectively at regular intervals proximal of stenosis at POD28. **G-H**: Morphometric analysis of pre-stenosis RCCA, via Mann-Whitney test, n=9-10/group. **G:** I/M area. **H:** I/M thickness ratio. **I**: Intimal area. **J**: Intimal thickness. *Data represented as: Mean ± SD. * p<0.05, **p<0.01, ***p<0.001, ****p<0.0001*

After graft harvest at POD28 and consecutive histology, the local GYY group had increased luminal graft diameters (**Fig. 4C,** representative images), confirmed by an increase in corrected luminal area (**Fig. S3A**) which correlated with the *in-vivo* ultrasound measurements on an individual mouse basis (**Fig. S3B**).

After quantification of the vein graft wall, we found that local GYY treatment decreased I/M+A area ratios with 56.7% (**Fig. 4D**) and resulted in a 44.8% reduction in I/M+A thickness ratios (**Fig. 4E**), signifying a strong attenuation of VGD after periprocedural treatment with GYY. Intimal area (**Fig. 4F**) and thickness (**Fig. 4G**) were also decreased combined with an increase in M+A area (**Fig. S3C**) and thickness (**Fig. S3D**). Overall, this pointed towards improved and beneficial adaption of the vein graft to its new arterial environment, as was shown by a limited inward remodeling response (**Fig. 4B-G**) and increased outward remodeling (**Fig. S3C-D**) after local GYY treatment.

A major hallmark of VGD and consecutive graft failure, is the proliferation and migration of VSMCs from medial and adventitial layers of the vein conduit into the intimal layer.[5] Interestingly, at POD28 there was a 30.6% decrease in intimal VSMCs (**Fig. 4H**) in the local GYY group (**Fig. 4I**) while there was no difference in proliferating VSMCs at this post-operative time-point (**Fig. 4J**), nor was there a difference in collagen deposition (**Fig. S3E**). This pointed towards attenuation of VSMC migration by local GYY treatment as an underlying mechanism for its therapeutic effect, we therefore examined the effects of GYY on VSMCs *in vitro*.

3.5 Periprocedural H_2S therapy limits VSMC migration and proliferation *in-vitro*.

To further investigate this phenotype, we isolated VSMCs from murine aortas and confirmed their origin by immunocytochemistry with alpha-SMC (**Fig. 5A**). Intracellular H_2S levels were measured with SF_7-AM upon 6hr GYY stimulation (**Fig. 5B**). Next, we tested whether GYY treatment would impair VSMC migration *in vitro* by either preconditioning VSMCs with GYY (6 hours, 250μM GYY) or stimulate them with GYY for the duration of the Boyden chamber assay (6 hours). Interestingly, VSMC transmigration was limited both by pre-conditioning (**Fig. 5E**, 6hrs pre-conditioning) and co-stimulating (**Fig. 5C, D**, peri-GYY) PDGF-BB stimulated VSMCs.

To rule out cell-death as an explanation for limited VSMC migration during/after GYY stimulation and to test the toxicity of our compound we next performed live-imaging on VSMC stained with proprium iodide (PI) during GYY supplementation in various concentrations. Most importantly, 250μM did not increase cell death, but of note was that even 1000μM of GYY did not prove toxic for VSMCs (**Fig. 5F)**. Since GYY did not have any detectable toxic effects on VSMC we next assessed whether it influenced metabolic activity/proliferation via an MTT assay. Proliferation was not different after short incubations with

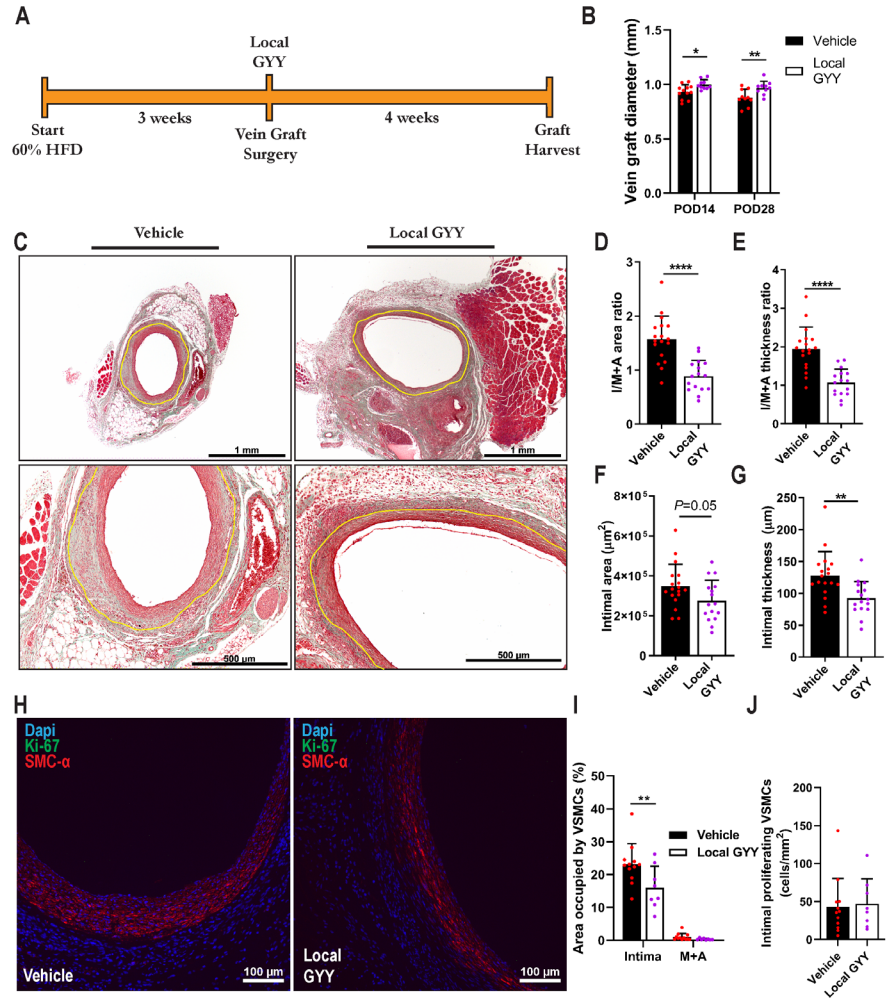


Figure 4. **Periprocedural local H_2S therapy protects from vein graft disease by attenuating intimal VSMC migration.**

A: Experimental outline with local GYY application during vein graft surgery. **B**: Ultrasound measurements of vein graft diameters at POD14 and POD28 by two-way ANOVA with Sidak's multiple comparisons test; n=10-12/group. **C:** Representative images of locally treated vein grafts at POD28 after Masson-trichome staining. Yellow lining indicating the intima-media+adventitia border. Scale bars 1mm/500μm as indicated. **D-G**: Morphometric analysis of vein grafts at POD28, n=16-18/group, student's t-test was performed unless indicated otherwise. **D:** I/M+A area ratios. **E**: I/M+A thickness ratios **F**: Intimal area. **G:** Intimal thickness. **H**: IHC for SMC-α + Ki-67. Scale bars are 100μm as indicated. **I:** Percentage of area occupied by SMC-α positive cells in intimal and M+A layers, via two-way ANOVA with Sidak's multiple comparisons test; n=8-12/group. **J**: Intimal co-localization of VSMC and Ki-67. *Data represented as: Mean ± SD. * p<0.05, **p<0.01, ****p<0.0001*.

GYY (**Fig. 5G**), while medium length incubation slightly amplified VSMC proliferation (**Fig. 5H**). At the 18hr timepoint however, GYY limited VSMC proliferation, especially at higher concentrations (**Fig. 5I**).

Lastly, since endothelial cells (ECs), next to VSMC, play an important role in both early- and late vascular remodeling[32], we repeated our analysis of the toxicity and metabolic effects of GYY but then on HUVECs. Interestingly, live-imaging with PI revealed a slight increase in cell survival compared to control conditions in HUVECs supplemented with 250µM or 1000µM GYY at T=18-19hrs (**Fig. 5J**), indicative of a potential cytoprotective effect of GYY on ECs. Further analysis of HUVEC proliferation via MTT assays (**Fig. 5K-M**) showed reduced metabolic activity/proliferation after longer periods of incubation (T=18hr) and high (1000µM) GYY concentration (**Fig. 5M**).

Taken together, these data suggest that a single local periprocedural administration of the H_2S donor GYY is able to attenuate VGD, and that this is at least partly through limiting VSMC migration and proliferation. Benefits from local GYY therapy in vascular remodeling could, parallel to limited VSMC migration/proliferation, possibly extend to increased cell survival and cytoprotection in ECs.

4. Discussion.

The prospect of translating short-term dietary preconditioning strategies into everyday surgical practice appears appealing as such approaches have been tested in certain patient populations. For example, in healthy kidney donors scheduled for transplant surgery, a two-week protein-calorie restriction diet was proven feasible in terms of compliance.[33] Furthermore, just 1 week of pre-operative caloric restriction diet was able to reduce intra-operative blood loss in patients undergoing liver resection.[34, 35] In the vascular surgery patient population however, with a predisposition towards unhealthy lifestyle choices and increased metabolic disease even a short-term change in dietary patterns may prove challenging from a DR-compliance perspective. In our recent pilot study only about 10% of appropriate patients were able to undergo a defined three-day dietary intervention prior to elective vascular surgery.[24]

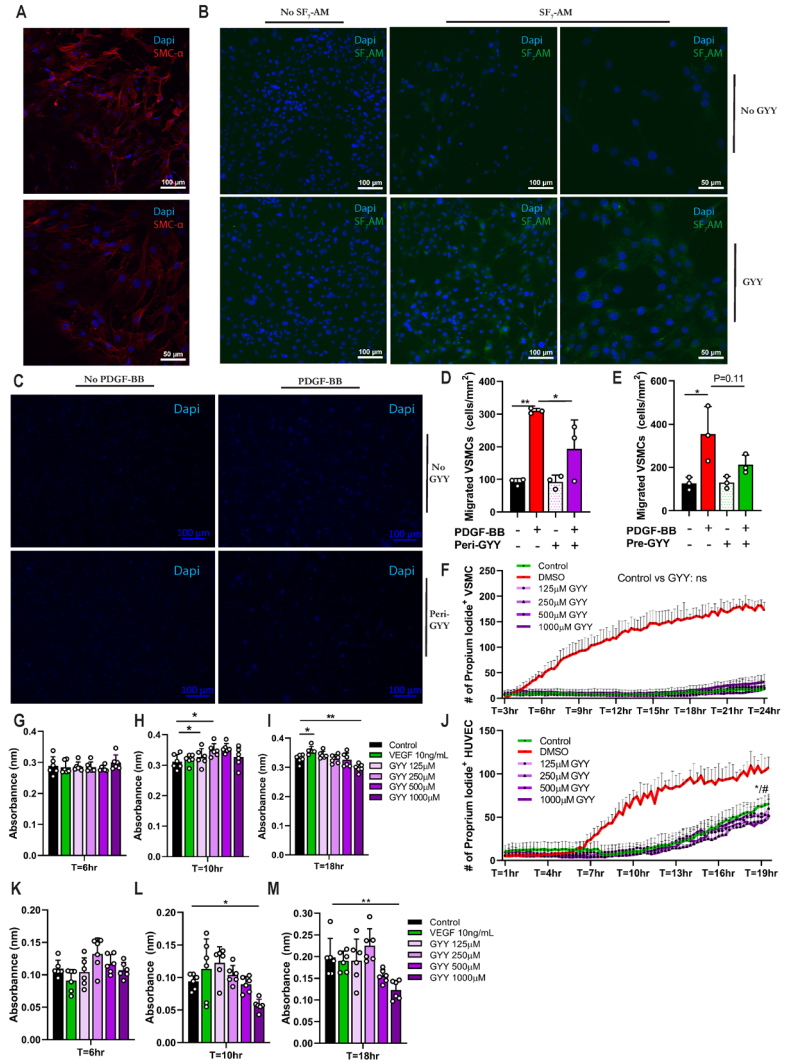


Figure 5. Periprocedural H_2S therapy limits VSMC migration in-vitro via

A: Representative immunocytochemistry image (20x & 40x magnification) of primary VSMCs after SMC-α staining. Scale bars 100µm and 50µm as indicated. **B:** VSMCs after 6hr treatment with 250µM GYY, with/without SF_7-AM incubation (20x & 40x magnification). Scale bars 100µm and 50µm as indicated. **C**: VSMCs after Transwell migration assay (6hrs in presence or absence of PDGF-BB and/or 250µm GYY). **D**: Quantification of Transwell migration assay. Number of migrated VSMCs normalized to per mm^2 after 6hrs PDGF-BB and/or 250µM GYY incubation in triplicates via one-way ANOVA with Sidak's multiple comparisons test. **E**: Quantification of Transwell migration assay after 6hrs of PDGF-BB incubation and/or 6hrs of 250µM GYY pre-treatment. *Data represented as: Mean ± SEM. * p<0.05, **p<0.01.*

Here we established a promising new approach to mitigate graft failure after bypass surgery by local delivery of the H2S donor GYY during the vein graft procedure. This local periprocedural therapy would circumvent the need for preconditioning via DR or pharmacological therapy (with H_2S-donors) and increase the potential for clinical translation. A one-time periprocedural application of this H_2S-gel onto the graft proved sufficient to limit inward graft remodeling, while parallelly improving vein graft outward adaptations. Furthermore, even when compared to systemic pre- or post-conditioning with GYY, local application had increased efficacy in attenuating VGD. Protection from VGD and vein IH notwithstanding, our H_2S-gel was additionally able to reduce arterial hyperplasia and improve arterial remodeling in a mouse model of arterial focal stenosis. Thereby bolstering the potential of this intervention beyond vein graft surgery, to extend towards both open and endovascular arterial procedures.

Previous research found that systemic treatment with H_2S donors, via IP injection, attenuated arterial remodeling after balloon angioplasty.[36] In a follow-up study the same group showed decreased VSMC proliferation and migration *in-vitro* as a result of exogenous H_2S therapy, due to downregulation of matrix-metalloproteinase-2.[22] And recently, a slow-releasing H_2S-peptide gel was developed that limited VSMC migration *in-vitro* and *ex-vivo* in transplanted human vein graft segments.[37] Here we show that local (exogenous) H_2S therapy limits intimal VSMC migration *in-vivo*, which in turn contributes towards protection from graft failure. (**Fig. 6**) This supports our previous work on the role of H_2S in vein grafts, where we found that endogenous upregulation of H_2S (via DR) protected from VGD, also partly via inhibition of the fibroproliferative response.[23] Whether exogenous H_2S therapy (and endogenous) also mitigates dedifferentiated VSMC migration or is involved in VSMC phenotype switching, both important hallmarks in the intimal hyperplastic response,[38] remains to be determined in future studies. However, H_2S is also known for its anti-inflammatory properties.[18] For example, systemic H_2S therapy decreased circulating tissue necrosis factor-α after transient aortic occlusion, implying a decrease in the inflammatory response after vascular injury.[39] It is likely that increased locally available H_2S not only directly inhibits VSMC migration, but also indirectly, by blocking the influx and activation of immune cells.

A possible concern in exogenous H_2S supplementation is its narrow therapeutic window, and the toxicity of H_2S in high concentrations.[40] We therefore opted for a slow-releasing H_2S donor (GYY4137) as opposed to the more conventional exogenous H2S treatment with the fast-releasing H2S donor NaHS. Since GYY continuously releases free H_2S molecules in low concentrations, we circumvented any potential issues of toxicity via oral administration. Furthermore, GYY being a prodrug, its parent molecule ZYJ112 has not shown any biological (or adverse) effects in-vitro or in-vivo, [41] which further reinforces the potential of GYY as a safe candidate for (oral) exogenous H_2S treatment.

Despite the translational promise this work holds, there are several limitations to be acknowledged. Firstly, this intervention was only tested on vein grafts and arteries at 28 days post-surgery, since in this model long-term protection cannot be established. Secondly, although we established protection from VGD at POD28, the effects of GYY during early vein graft remodeling are unknown. We hope that this work will incite future research on the effects of local H_2S therapy on the different cell types (VSMC, endothelial cells, leukocytes) that play a major role in facilitating or accelerating this remodeling response. Thirdly, only a single concentration of GYY was tested, therefore the most efficacious and optimal dose of GYY in VGD/arterial remodeling is unknown. Lastly, although a high-fat diet was employed to mimic the vascular surgery patient population, other factors were not accounted for (age, gender, underlying co-morbidities).

In short, we developed and tested a H_2S releasing gel that can be applied locally during the procedure, and that is capable of attenuating both vein graft failure and arterial remodeling. Future directions should focus on exploring its potential in vascular access surgery and endovascular interventions.

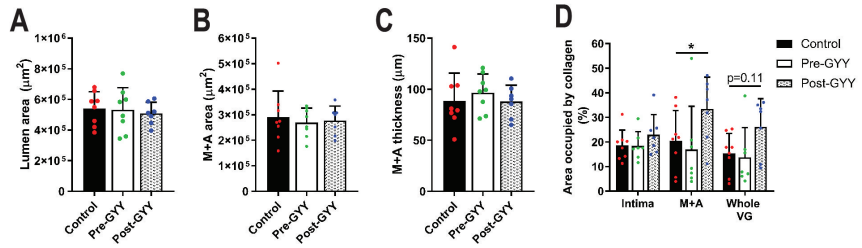
5. Funding.

This work was supported by an American Heart Association Post-Doctoral Grant [#19POST34400059] and grants from Foundation 'De Drie Lichten', Prins Bernhard Cultural Foundation and Michael-van Vloten Foundation to P.K.; the Harvard-Longwood Research Training in Vascular Surgery NIH T32 Grant 5T32 HL007734-22 to KT; American Heart Association Grant-in-Aid 16GRNT27090006; National Institutes of Health, 1R01HL133500 to C.K.O.; and NIH(AG036712, DK090629) and Charoen Pokphand Group to J.R.M.

6. Acknowledgements.

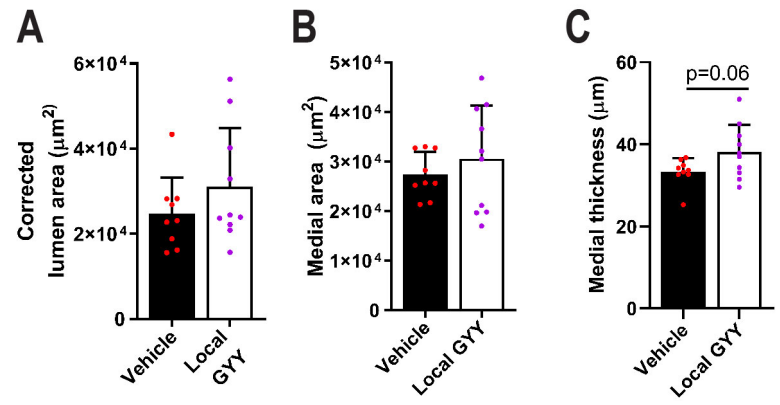
P.K. provided funding, conceived of experimental designs, performed experiments and wrote the manuscript. M.T. performed surgeries, processed vein grafts and analyzed histology. M.R.V. conceived of experimental designs, performed surgeries and advised with P.H.Q. on analysis of vein grafts. K.T., M.R.M. and S.J.M. assisted with animal care and in-vivo studies. C.M., J.W.J., H.A.B, M.L.B and T.S. assisted with ex-vivo and in-vitro experiments. S.P. assisted with histomorphometric analysis of vein grafts. J.R.M. and C.K.O. provided funding, conceived of experimental designs and supervised the project. We thank M.L. van der Bent for her expertise and support with live imaging of primary cells.

Conflict of Interest: none declared.



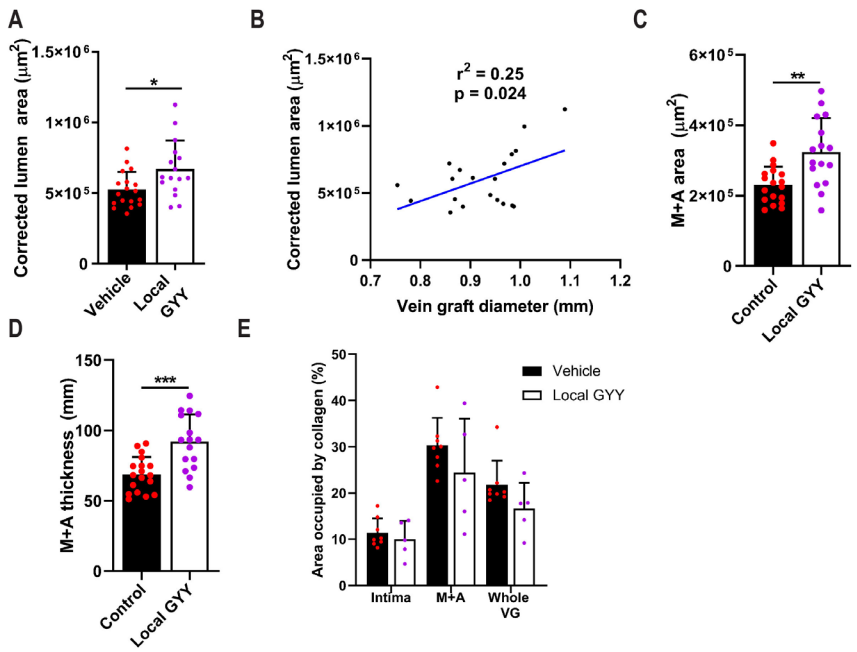
Supplemental 1, related to Figure 1.

A: Corrected lumen area in control, pre- and post-GYY treated vein grafts at POD28. **B**: M+A area. **C**: M+A thickness. **D:** Percent of intimal and M+A layers and whole VG occupied by collagen via two-way ANOVA with Turkey's multiple comparisons test.



Supplemental 3, related to Figure 3.

A: Averaged corrected lumen area in locally treated carotid arteries at POD28 after focal stenosis procedure. **B**:M+A area. **C**: M+A thickness. *Data is represented as Mean ± SD*



Supplemental 4, related to Figure 4.

A: Corrected lumen area in vehicle and local GYY treated vein grafts at POD28. **B:** Correlation between corrected luminal area after histology and vein graft diameter ultrasound measurements *in vivo*, both at POD28. **C**: M+A area. **D:** M+A thickness. **E**: Percentage of vein graft layers occupied by collagen. *Data represented as Mean ± SD. * p<0.05, **p<0.01, ***p<0.001*.

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