Short-term pre-operative dietary restriction in vascular surgery

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Chapter 3.

Short-Term Pre-Operative Protein Restriction Attenuates Vein Graft Disease Via Induction of Cystathionine Y-Lyase.

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Abstract.

Aims: Therapies to prevent vein graft disease, a major problem in cardiovascular and lower extremity bypass surgeries, are currently lacking. Short-term preoperative protein restriction holds promise as an effective preconditioning method against surgical stress in rodent models, but whether it can improve vein graft patency after bypass surgery is undetermined. Here, we hypothesized that short-term protein restriction would limit vein graft disease via upregulation of cystathionine-gamma-lyase and increased endogenous production of the cytoprotective gaseous signaling molecule hydrogen sulfide.

Methods and Results: Low-density lipoprotein receptor knockout mice were preconditioned for one week on a high-fat high-cholesterol diet with or without protein prior to left common carotid interposition vein graft surgery with caval veins from donor mice on corresponding diets. Both groups were returned to a complete high-fat high-cholesterol diet postoperatively, and vein grafts analyzed 4 or 28 days later. A novel global transgenic cystathionine-gamma-lyase overexpressing mouse model was also employed to study effects of genetic overexpression on graft patency. Protein restriction decreased vein graft intimal/media+adventitia area and thickness ratios, intimal smooth muscle cell infiltration 28 days postoperatively, and neutrophil transmigration 4 days postoperatively. Protein restriction increased cystathionine-gamma-lyase protein expression in aortic and caval vein endothelial cells and percentage of lung endothelial cell producing hydrogen sulfide. The cystathionine-gamma-lyase inhibitor propargylglycine abrogated protein restriction-mediated protection from graft failure and the increase in hydrogen sulfide-producing endothelial cells, while cystathionine-gamma-lyase transgenic mice displayed increased hydrogen sulfide production capacity and were protected from vein graft disease independent of diet.

Conclusion(s): One week of protein restriction attenuates vein graft disease via increased cystathionine-gamma-lyase expression and hydrogen sulfide production, and decreased early inflammation. Dietary or pharmacological interventions to increase cystathionine-gamma-lyase or hydrogen sulfide may thus serve as new and practical strategies to improve vein graft durability.

1. Introduction.

Lifestyle choices such as diet contribute to the development and progression of atherosclerosis and cardiovascular disease [1, 2] requiring lower-extremity or coronary bypass surgery.[3, 4] Harvesting a suitable venous conduit and transplanting it into an arterial circuit induces a cascade of biologic events including ischemia-reperfusion injury, oxidative stress and acute biomechanical perturbations such as increased wall shear stress [5,6]. The subsequent adaptation to an arterial environment continues to stimulate endothelial cells following transplantation,[7] in turn upregulating various pro-inflammatory surface markers such as vascular cell adhesion molecule and intercellular adhesion molecule [8, 9]. Circulating leukocytes then attach to adhesion molecules and transmigrate across the luminal wall [8, 10], initiating a cascade leading to intimal hyperplasia (IH) and vein graft occlusion [11]. Although autogenous vein grafts remain superior to prosthetic conduits for lower-extremity bypass grafting [12, 13], nearly 40% of autogenous vein grafts will develop IH and subsequently occlude over time, leading to re-interventions, amputation, myocardial infarction or death [13]. While the prospect of engineered decellularized vascular grafts appears promising as a conduit option,[14] currently the most durable vascular graft for peripheral arterial bypass remains the autogenous vein graft.[13, 15] Furthermore, patients with multivessel coronary artery disease are best treated with coronary artery bypass grafting (CABG), in which >90% necessitate both arterial and vein grafts as bypass conduits to revascularize ischemic myocardium [16, 17]. Yet, no significant therapies have been developed to prevent vein graft disease.

Defining mechanisms of IH and employing novel strategies to attenuate the fibroproliferative response are thus critical in order to improve revascularization surgery outcomes. Emerging basic science and clinical trials point to the promise of brief pre-operative dietary restriction as an intervention with the potential to improve surgical outcomes, [18, 19] even in elective cardiovascular surgery patients who have high rates of post-operative morbidity and mortality [20]. Such dietary interventions are rooted in the field of aging and lifespan extension by nutrient/energy restriction, [21, 22 23] which was first shown to reduce the incidence of cancer in rodents over a century ago, [24] and include reducing total calorie intake, intermittent fasting, or dilution of dietary components such as protein or amino acids [25].

Recently we reported numerous benefits of dietary restriction preconditioning in pre-clinical rodent surgical models of ischemia-reperfusion injury to various organs, revascularization following femoral ligation, and intimal hyperplasia following focal stenosis [26-30], [31] without decelerating the post-intervention wound healing process[32]. On a mechanistic level, short-term dietary restriction interventions induce pleotropic and rapid changes in metabolism [27, 33, 34] and immune function [35] that in the surgical context can be anti-inflammatory and promote stress resistance [23]. For example, dietary preconditioning against hepatic ischemia reperfusion injury or femoral ligation in mice involves upregulation of CGL and endogenous production of H_2S [26] [26], a dynamic mediator of vascular homeostasis [36, 37]. At least two other enzymes, cystathionine β-synthase (CBS) and 3-mercaptopyruvate sulfur-transferase, can also generate H_2S. Within a vessel, CGL is the most abundant H_2S-producing enzyme in endothelial cells (ECs),[38] and ECs lacking CGL display increased monocyte adhesion and susceptibility to atherogenesis[39]. H_2S exerts proangiogenic [40] and cytoprotective effects on ECs [41] and limits leukocyte attachment to the endothelium [42]. It is also a potent vasodilator [43] and inhibitor of vascular smooth muscle cell proliferation,[44] thereby showing therapeutic potential in vein graft disease.

While reducing total calorie intake prior to elective surgery could prove difficult for patients with cardiovascular disease, similar benefits have been achieved in preclinical models via restriction of protein or sulfur amino acids in rodent models of renal or hepatic ischemia-reperfusion injury and femoral ligation [26, 29, 45, 46]. Here, we tested the hypothesis that dietary preconditioning with an isocaloric protein-free diet for one week prior to surgery will attenuate vein graft IH via increased CGL expression and H_2S production.

2. Materials & Methods.

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 or A4752-01) and in accordance with the NIH guidelines. Surgical vein graft experiments were performed on 10-12-week low density lipoprotein receptor knockout ($LDLr^{-\backslash-}$) mice (male, B6.129S7-$Ldlr^{tm1Her}$, Stock No:002207, Jackson Laboratory, n=10-18 per group), CGL^{tg} mice (male, 10-12 week, hemizygous for bacterial artificial chromosome containing CGL described in more detail below on a C57BL/6J background vs. littermate non-transgenic controls, n=5-8 per group) or C57BL/6J (male, 10-12 weeks old, Stock No: 000664, Jackson Laboratory). For dietary preconditioning baseline studies either male $LDLr^{-\backslash-}$ or C57BL/6J (male, 10-12 weeks old, Stock No: 000664, Jackson Laboratory) mice were used. Mice were housed 4-5 per cage and maintained on a 12-hour light-dark cycle at 22°C with 30-50% humidity.

2.2 Construction of CGL BAC transgenic model.

BAC RP24-344N5 containing the CGL locus from the RPCI-24 BAC library on a male C57BL/6J background was obtained from CHORI. The highly active Ankrd13c promoter located approximately 20kb upstream and antisense to the CGL promoter was removed by first inserting a cassette containing a NotI-FRT-kan/neo-FRT restriction site ~4 kb upstream of the Ankrd13c promoter by RedET recombination, and then digesting the resulting BAC with NotI. This released a larger 157 kb fragment containing Cgl with 18 kb upstream and over 100 kb downstream of the Cgl CDS, but with no other exon sequences present. This fragment was isolated by pulse field gel electrophoresis, purified and injected into C57BL/6J oocytes. A single founder line was identified by PCR using primers 2981 (CTH Common F: TGGGACAGCTCTTCTCCCTTA), 2982 (CTH Tg R: GCAGAATTCCACCACACTGGACTA) and 2983 (CTH WT R: TTCTGTGAGGAGGGAGCCAT) resulting in a 577 bp control band from the WT allele, and a 369 bp band from the BAC transgene. Based on mRNA and protein analysis, we predict insertion of at least two copies of the BAC into an unknown location in the genome. The BAC transgene array was maintained by breeding non-transgenic to hemizygous BAC-transgenic animals and using hemizygous BAC-tg vs. WT littermate controls in all experiments.

2.3 Diets.

Upon arrival, mice were acclimated to the animal facility for three days, and subsequently fed a complete high-fat high-cholesterol (HFHC) diet (20% Kcal protein, 40% Kcal carbohydrates, 40% Kcal fat, 1.25% cholesterol, Research Diets, New Brunswick, NJ, Diet D12108C) on an *ad libitum* basis in order to mimic the hyperlipidemic status of the typical vascular surgery patient with occlusive arterial disease. Following three weeks of HFHC feeding, cages were randomized into the following groups: HFHC donors, HFHC recipients, PR-HFHC donors and PR-HFHC recipients. PR-HFHC diets consisted of a HFHC base in which all protein was isocalorically replaced with carbohydrates (0% Kcal protein, 60% Kcal carbohydrates, 40% Kcal fat, 1.25% cholesterol, Research Diets, New Brunswick, NJ, Diet D16082403). These diets were continued for one

week prior to surgery. Vein grafts were performed in PR or HFHC recipient mice with thoracic inferior vena cava's (IVC) from donor mice treated with the same dietary intervention (either complete HFHC or PR-HFHC), or to test the effects of donor vs. recipient only the donor or recipient mouse was fed PR-HFHC for 7 days. Immediately following surgery all groups resumed the complete HFHC diet. Mice were weighed weekly.

In a separate experiment, LDLr$^{-/-}$ mice were injected with either vehicle (0.9% sodium chloride) or the CGL inhibitor DL-propargylglycine (PAG, Sigma Aldrich, 10-20mg/kg intra-peritoneal daily for 7 days) during a week of dietary intervention (PR + vehicle, PR + PAG) and were either euthanized after 7 days of dietary intervention or were subjected to vein graft surgery and euthanized 28 days after surgery.

2.4 Vein Graft Surgery.

Interposition vein graft surgery was performed as previously described.[47] Briefly, the mouse was anaesthetized with 3-5% isoflurane and maintained under nose-cone with continuous 1.5-3% isoflurane inhalation. Hair was removed and a midline neckline incision was performed. The right common carotid artery (CCA) was dissected, ligated midway with an 8-0 nylon suture and then divided. The CCA was clamped proximally and distally and a polyetheretherketone cuff was placed on the proximal and distal arterial ends. The arterial lumen was then everted over the cuffs. The thoracic IVC from a donor mouse was harvested, sleeved over the cuffs and secured by 8-0 nylon sutures. Proximal and distal clamps were removed and patency of the graft was confirmed by visual inspection. The incision was closed with 6-0 vicryl sutures. Postoperatively animals received warm lactate ringer solution (0.5-1.0ml, subcutaneous) and buprenorphine (0.1mg/kg, subcutaneous).

2.5 Duplex Ultrasound Biomicroscopy.

High-resolution ultrasonography was performed *in vivo* on mouse vein grafts at day 28 using a Vevo 2100 imaging system with 18- to 70-MHz linear array transducers (VisualSonics Inc., Toronto, ON, Canada). Mice were anesthetized with inhaled isoflurane, and body temperature was maintained using a 37°C heated stage. M-mode was used for vessel cross-sectional dimensions. Three luminal axial images were performed (proximal, distal & mid vein graft) and mean vessel luminal diameters were calculated.

2.6 Vein Graft Analysis.

Experimental endpoints were analyzed at baseline, day 4 or day 28 after surgery. Whole blood was obtained via percutaneous heart puncture in anesthetized mice, which were then perfused with lactate Ringers solution for 3 minutes and perfusion-fixed with 10% formalin for 3 minutes. For harvest of vein grafts, the neck was opened, and the graft was harvested en-bloc and fixed in 3% formaldehyde in PBS for 24 hours or frozen in OCT for immunohistochemistry. IVCs and thoracic aortas were used for isolation of primary endothelial cells or flash frozen for isolation of protein/RNA.

2.7 Tissue Processing and Histology.

Vein grafts embedded in O.C.T. compound (Tissue-Tek) were cut in 5µm sections by cryotome. After fixation in formalin for 24 hours, vein grafts were transferred to 70% ethanol and then embedded into paraffin blocks. Vein grafts were cut using a microtome in 5µm thick cross sections at regular 200µm intervals starting from the proximal cuff. Cross sections were mounted on slides and Masson trichome histology staining was performed. In short, slides were deparaffinized to 95% ethanol, stained in 5% picric acid (in 95% ethanol) for 3 minutes, washed with tap water, stained in working Harris Hematoxylin Solution (Fisher Scientific, cat# 245-678) 3 minutes, washed with tap water, stained with 1% Biebrich Scarlet in 1% acetic acid (Fisher Scientific, cat# A38S-500) for 3 minutes, rinsed in distilled water, 5% Phosphomolybdic/Phosphotungstic acid solution for 1 minute, 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, then in 1% acetic acid solution (Fisher Scientific, cat# A38S-500) for 2 minutes. Slides were dehydrated with xylene and mounted with cover glass using permount.

2.8 Immunohistochemistry.

Slides were pre-incubated in a vacuum oven for 30 minutes at 65ºC. Then deparaffinization was performed via consecutive washes in xylene (3x5 minutes), 100% ethanol (3x5 minutes), 75% ethanol (1x5 minutes), 50% ethanol (1x5 minutes). For antigen retrieval, slides were incubated in citrate buffer (Abcam, ab94674) for 30 minutes at 96ºC. After cool down to room temperature (RT), slides were pre-incubated in a 10% goat serum (Life Technologies, #50062Z), 0.3M glycine (Ajinomoto, #R015N0080039) solution for 1 hour at RT. Consecutive incubation with primary antibodies for SMC-α (mouse anti-mouse, Abcam, ab7817), Neutrophil-elastase (rabbit anti-mouse, Abcam, ab68672), CGL (rabbit anti-mouse, Abcam, ab151769), CBS (rabbit anti-mouse,

Abcam, ab135626), SMC-α (mouse anti-mouse, Abcam, ab7817) and Ki-67 (rabbit anti-mouse, Abcam, ab16667) fluorescent double staining overnight at 4ºC. The following day slides were washed in PBS + tween (0.05%) solution (3x5 minutes). For fluorescent IHC protocols tissues were then stained with secondary antibodies Alexa Fluor 488 (goat anti-rabbit, AA11034), Alexa Fluor 568 (goat anti-rat, A-11077), Alexa Flour 568 (goat anti-rabbit, A-11011) or Alexa Fluor 647 (goat anti-mouse, A32728) for two hours at RT. After wash with PBS + Tween (3x5 minutes), slides were mounted with DAPI (Vector, CB-1000) and imaged. In conventional IHC, after binding primary antibodies with secondary biotinylated goat anti-rat (Vector Laboratories, BA-9401), goat anti-mouse (Vector Laboratories, BA-9200) or goat anti-rabbit (Vector Laboratories, BA-1000) antibodies for two hours at RT, slides were incubated with ABC-complex (Vectastain, PK-7100) for 30 minutes at RT. Slides were then washed with PBS (3x5 minutes) and incubated with a DAB peroxidase substrate kit (Vector, SK-4100) for various time-periods depending on the manufacturers protocol. Slides were then dehydrated via consecutive washes in 50% Ethanol (1x5 minutes), 70% Ethanol (1x 5 minutes), 100% Ethanol (3x5 minutes), Xylene (3x5 minutes), mounted with Permount (Fischer Scientific) and imaged. For IHC on frozen tissue embedded in OCT slides were defrosted at RT, fixed in 4% paraformaldehyde, washed in phosphate buffered saline (PBS) and then incubated in antigen retrieval solution (PBS, 0.2% Triton-X). Slides were incubated in 10% goat serum then incubated with primary antibody (neutrophil anti-elastase) overnight at 4ºC. The following day slides were washed in PBS + tween (0.05%) solution (3x5 minutes), incubated with the secondary antibody Alexa Flour 568 (goat anti-rabbit, A-11011) for 2 hours at RT. Slides were subsequently washed with PBS, stained with DAPI and imaged.

2.9 Histology and Immunohistochemistry Analysis.

Slides were imaged using a Zeiss Axio A1 microscope (Carl Zeiss) and histomorphometric and immunohistochemistry analysis was performed in ImageJ 1.51p (Java 1.8.0_66). All histological and immunohistochemical analysis was performed while observer was blinded. Histology analysis consisted of 5 slides per graft, 200μm apart beginning from the proximal cuff, with 3 cross-sections per slide. Luminal, intimal, medial and adventitial areas and circumferences were measured from each cross-section, mean value was calculated per slide and consecutively per vein graft. Intimal area and thickness, medial+adventitial (M+A) area and thickness, intimal versus medial+adventitial (I/M+A) area and I/M+A thickness ratios were calculated. For collagen content of intimal and M+A layers, Masson trichome stained slides were processed in ImageJ by color-deconvolution and percentage of green stained area compared to total area was calculated. Immunohistochemistry analysis consisted of 1 slide per venous graft, at a distance of 400μm from the proximal cuff. For smooth muscle cell (SMC-α) proliferation, the intimal and (M+A) area occupied by SMC-α stained cells was calculated via the color deconvolution function in ImageJ. Cells co-localizing both SMC- α and Ki-67 signal were designated as proliferating SMC and manually counted. Neutrophil-elastase positive cells were manually counted employing ImageJ and normalized by area (in mm^2) of each vein graft layer.

2.10 qPCR/mRNA Analysis.

Whole tissue samples were homogenized in 5 volumes of RNA-BEE (Tel Test B labs, # 173107-521) using a tissue homogenizer, after which samples were centrifuged at 12,000xG at 4°C for 10 minutes. Supernatant was then transferred to a 1.5 mL Eppendorf tube, combined with 200 μL chloroform (Sigma-Aldrich, #288306-1L) and vortexed. After 5 minutes of incubation on ice, samples were centrifuged at 12,000 x *g* at 4°C for 15 minutes. The aqueous layer was collected in a fresh tube on ice and 1 volume of isopropanol was added per sample, then centrifuged (12,000 x *g* at 4°C for 10 minutes). After aspiration of supernatant, 1 mL 75% ethanol was added to each tube, vortexed and then centrifuged (12,000 x *g* at 4°C for 10 minutes). After aspirating the supernatant, RNA pellets were left to dry until partially translucent. Pellets were resuspended in 20μL RNAase free H_2O and stored at -80°C. cDNA was synthesized using Verso cDNA kit (Thermo #AB-1453/B) according to manufacturer's instructions. qRT-PCR was then performed using SYBR green master mix (BioRad #1725274) and fold changes were calculated using the delta delta CT method with each sample normalized to B2m and 18s housekeeping genes. 18s primers used were F (GTAACCCGTTGAACCCCATT) and R(CCATCCAATCGGTAGTAGCG), B2m primers used were F(CGGCCTGTATGCTATCCAGA) and R(GGGTGAATTCAGTGTGAGCC), Cgl primers used were F(TTGGATCGAAACACCCACAAA) and R(AGCCGACTATTGAGGTCATCA) and Atf4 primers were F(TCGATGCTCTGTTTCGAATG) and R(AGAATGTAAAGGGGGCAACC).

2.11 Intracellular H2S Determination in Endothelial Cells by Flow Cytometry.

Due to low endothelial cell yield from murine vessels (e.g. aorta, IVC, carotid artery) lungs were used to a obtain a single cell suspension as previously described.[48] Briefly, the organ was harvested and gently minced using a scalpel, placed into media (DMEM, Gibco #11965-092) and placed into digestion buffer (DMEM Media, Gibco #11965-092) with 2mg/mL collagenase II & IV (Gibco

#9001-12-1) for 30 minutes at RT under continuous horizontal shaking (300 rpm), strained through a 100µm cell strainer, and then centrifuged for 5 minutes at 1200 rpm and the supernatant discarded. The resulting cellular pellet was washed with antibody binding buffer (ABB) (2mM EDTA, 1% FBS/CCS, 0.5% BSA, in dPBS) and centrifuged for 5 minutes at 1200 rpm, decanted and cells were counted using a hemocytometer and split into respective tubes for staining for flow cytometric analysis. Cells were stained for 30 minutes in the dark at 4 ºC with CD31 APC (1µl/1x10^6 cells, Miltenyi Biotec, #130-102-571) for endothelial cell staining and P3 to measure intracellular H_2S (10µM final concentration, Millipore Sigma #595504). Cells were then fixed in 2% paraformaldehyde, washed and analyzed using a Fortessa Flow Cytometer (BD Biosciences, San Jose, CA, USA). Appropriate controls consisted of an unstained control for compensation and gating as well as single stained CD31 and single stained P3 samples. CD31+ cells were used as a stopping gate for events collected by the flow cytometer (10,000 CD31+ cells) to ensure robust endothelial cell counts.

2.12 Western Blotting from Vessel and Isolated Vessel Endothelial Cells.

To evaluate whole vessel protein expression following one week of protein restriction compared to control diet, thoracic aortas were surgically dissected (perivascular fat removed) and frozen in liquid nitrogen. Whole aortas were homogenized with passive lysis buffer (Promega #E1941), normalized for protein content using a BCA assay, boiled with SDS loading buffer and separated by SDS-PAGE. Proteins were transferred to a PVDF membrane and blotted for CGL (Abcam, ab151769) and CBS (Abcam, ab135626). Aortas from $CGL^{-/-}$ mice were used as a specificity control for the CGL antibody. To assess protein levels from vessel endothelial cells, aortas and IVCs were digested for 30 minutes at 37ºC in collagenase/dispase digestion buffer (200U/mL collagenase type II Gibco #17101015, Elastase Promega #V1891, 1:2 in DMEM) under continuous horizontal shaking (300 rpm). After 30 minutes, an equal volume of complete DMEM with 10% FBS (Thermo Fisher #10082147) was added, and the cell mixture was passed through a 100µm cell strainer, centrifuged (5 minutes, 1200 rpm 4ºC) and digestion and centrifugation were repeated as above. After supernatant removal, the cell pellet was resuspended in ABB and consecutively incubated with 10 µL FC blocker for 10 minutes, 10µL APC conjugated CD31 (Miltenyi Biotec, #130-102-571) for 15 minutes, 20µL selection cocktail for 15 minutes, and then magnetic particles (10µL) were added. The tube was incubated in a cell separation magnet (Stemcell technologies, #18000) for 5 minutes, supernatant removed, and the pellet resuspended in 2.5 mL ABB.

After repeating three magnet incubations totaling 15 minutes, the isolated endothelial cells were resuspended in passive lysis buffer (Promega #E1941) and western blot was performed as described above.

2.13 Lead Acetate Method for H2S Production Capacity Measurement.

H_2S production capacity was measured using fresh or frozen tissue homogenized in passive lysis buffer as previously described.[49] Briefly, tissue homogenate was normalized for protein content and supplemented with 10mM cysteine and 1mM pyridoxal phosphate (PLP) in PBS in a 96 or 384 well plate. Lead acetate detection paper was placed on top of the plate and incubated for 2–4 hours at 37°C until lead sulfide darkening of the paper occurred.

2.14 Plasma Lipid Measurement.

Circulating plasma lipids were measured following one-week of dietary intervention using the Piccolo Lipid Panel Plus and the Piccolo Xpress chemistry analyzer following the manufacturer's instructions (Abaxis, Germany).

2.15 Statistical Analyses.

Data are expressed as mean ± standard error of the mean (mean ±SEM). Normality testing was performed using Shapiro-Wilk; normally distributed data was analyzed by Student's T-test, one-way or two-way ANOVA, and non-normally distributed data by Mann-Whitney or Kruskal-Wallis test using GraphPad Prism (7.0b).

3. Results.

3.1 Short-Term Protein Restriction Attenuates Vein Graft Disease.

We first examined whether PR attenuates intimal hyperplasia (**Fig. 1A,** schematic of IH development) in a validated surgical model of vein graft disease (donor vein graft harvested from separate mouse) using $LDLr^{-/-}$ mice fed a HFHC diet for three weeks to model the hyperlipidemic state of patients with arterial occlusive disease. One week prior to surgery, the experimental group (donors and recipients) were preconditioned on an isocaloric HFHC diet lacking protein (PR-HFHC) while the control mice were maintained on the complete 20% protein HFHC diet. Immediately after surgery, both groups resumed feeding on complete HFHC diet (**Fig. 1B,** dietary intervention). During the dietary intervention, PR-HFHC mice lost approximately 20% of their starting body weight despite no significant difference in food intake per gram of bodyweight

or per cage (**Supplemental 1A-C)**. Circulating lipid profiles of mice were unchanged as a function of protein restriction in this hyperlipidemic mouse model (**Supplemental D-F**).

Vein graft wall adaptations assessed via histo-morphometric vessel analysis at 28 days postoperatively showed significant beneficial effects of dietary preconditioning (**Fig. 1C**). Intimal/medial + adventitial (I/M+A) area ratios ($p<0.0001$. **Fig. 1D**) and thickness ratios ($p<0.0001$, **Fig. 1E**) were significantly lower in the PR-HFHC group compared to control group. Intimal area was significantly reduced ($p=0.0294$, **Fig. 1F)** while intimal thickness trended lower (**Fig. 1G)** in the PR-HFHC group. This was concomitant with an increase in M+A area ($p<0.0001$, **Fig. 1F**) and thickness ($p<0.0001$, **Fig. 1G**) in the PR-HFHC group. Taken together, these data demonstrate that short-term preoperative PR attenuates vein graft disease in a rodent model.

3.2 Preoperative Protein Restriction Limits Smooth Muscle Cell Migration and Inhibits Leukocyte Transmigration.

Endothelial cell damage and consequent influx of circulating inflammatory cells are hallmarks of early vein graft adaptations, resulting in intimal hyperplasia and loss of graft patency via a fibroproliferative response of SMC and migration into the intimal layer within 28 days (**Fig. 2A-B**)[50]. Preconditioning on PR significantly reduced the percentage of SMC in the intima ($p=0.0466$, **Fig. 2B**) despite a trend toward increased number of proliferating cells in this layer (**Fig. 2C**). PR grafts showed no difference in collagen content vs. control graft in the intimal or M+A layers **(Fig. 2D)**.

To investigate early vein graft adaptations giving rise to a differential SMC fibroproliferative response between diet groups, grafts were harvested on post-operative day 4. PR-HFHC fed mice lost about 20% of their starting weight during the dietary intervention, but as early as post-operative day 4 there were no differences in body weight between the dietary groups (**Supplemental G**). No differences were observed in percent area occupied by SMC or Ki-67 positive cells in intimal and adventitia + perivascular adipose tissue (PVAT) layers between dietary preconditioning regimens at this early time point after surgery (**Fig. 2E-G**). However, neutrophil anti-elastase staining was significantly reduced upon PR relative to controls in the intimal layer ($p=0.0184$, **Fig. 2H-I)**, adventitial + perivascular adipose tissue (PVAT) layer ($p=0.0077$, **Fig. 2I)** as well as in the total vein graft wall ($p=0.0146$, **Fig. 2I)** consistent with an overall decrease in

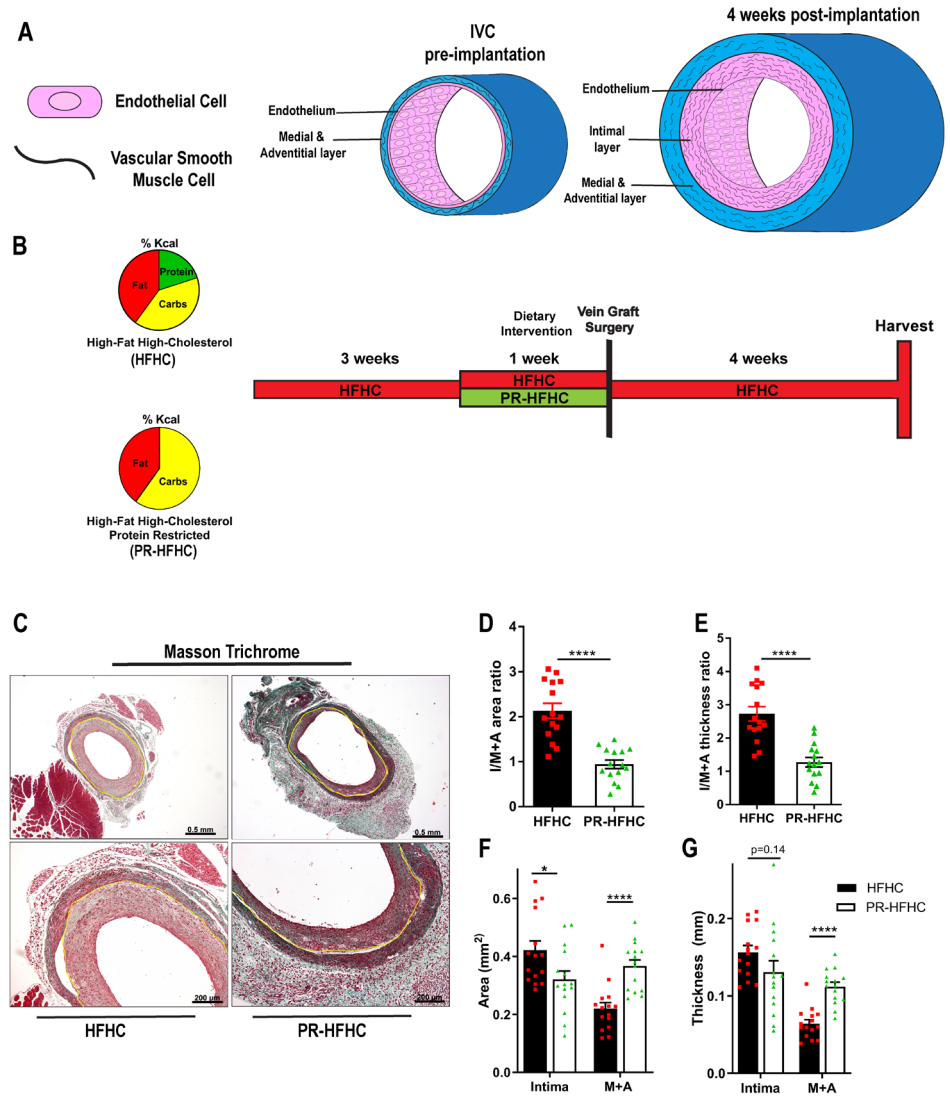

Figure 1. Short-Term Protein Restriction Attenuates Vein Graft Disease.
A: Schematic representation of the development of intimal hyperplasia 4 weeks after implantation of a vein graft (inferior vein cava, IVC) from a separate donor mouse. **B:** Schematic representation of diets (left) and dietary alterations (right) during the 3 week run-in, 1 week preconditioning and 4 week post-operative periods. All mice were subjected to a 3-week run-in period on a HFHC diet. **C**: Representative images of Masson trichome-stained vein grafts 28 days after surgery. Boundary between intimal (I) and medial + adventitial (M+A) layers is traced in yellow. Scale bars = 0.5mm or 200µm as indicated. **D-G**: Vein grafts assessments 28 days after surgery in HFHC vs. PR-HFHC preconditioned mice as indicated; n=15/group. **D**: I / M+A area ratios (2.1 ± 0.2 vs. 0.9 ± 0.1, $p<0.0001$, Student's T test). **E:** I / M+A thickness ratios (2.7 ± 0.2 vs. 1.2 ± 0.1, $p<0.0001$, Student's T test). **F:** Intimal thickness (0.156mm ± 0.009 vs. 0.131mm ± 0.015) and M+A thickness (0.064mm ± 0.005 vs. 0.112mm ± 0.006, $p<0.0001$, Student's T test). **G:** Intimal area ($0.422mm^2 \pm 0.03$ vs. $0.320mm^2 \pm 0.029$, $p=0.0294$, Mann Whitney test) and M+A area ($0.22mm^2 \pm 0.021$ vs. $0.367mm^2 \pm 0.021$, $p<0.001$, Student's T test). All data expressed as mean ± SEM; *$p<0.05$, ****$p<0.0001$.

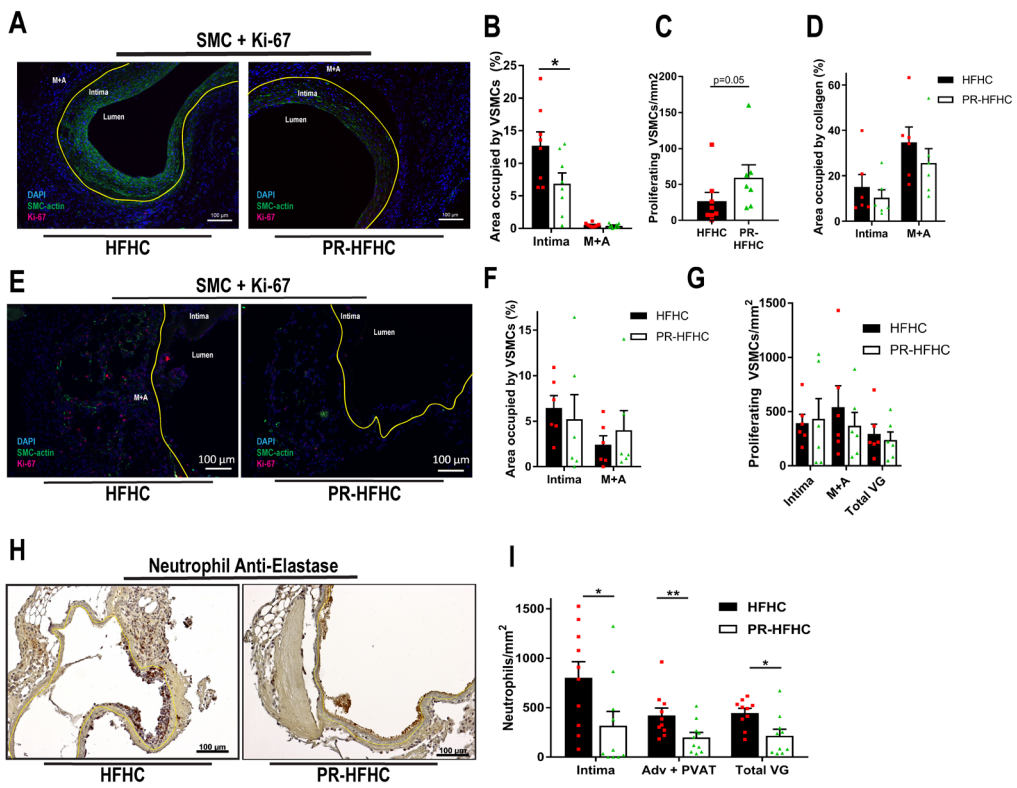

Figure 2. Preoperative Protein Restriction Limits Smooth Muscle Cell Migration and Inhibits Leukocyte Transmigration.

A-I: Assessment of intimal hyperplasia and neutrophil infiltration in vein grafts 28 days **(A-D)** or 4 days **(E-I)** after surgery in LDLr$^{-\backslash-}$ mice preconditioned on the indicated diets; n=5-10/group. **A:** Representative images of SMC-α (green), Ki-67 (red) and DAPI (blue) stained vein grafts. Included are lumen, intima and M+A as histological landmarks and the intima/M+A border is illustrated by yellow lining. **B:** SMC-α positive cells in intimal layer (12.7±2.1 vs. 6.9±1.6; p=0.0446, Student's T test) or M+A layer expressed as a percentage of area occupied. **C:** Number of proliferating SMC-α and Ki-67 double positive cells in the intimal layer per mm^2 (Student's T test). **D:** Percentage of intimal and M+A area occupied by collagen. **E:** Representative images of SMC-α (green), Ki-67 (red) and DAPI (blue) stained vein grafts from LDLr$^{-\backslash-}$ mice preconditioned as indicated and analyzed on post-operative day 4. Included are lumen, intima and M+A as histological landmarks and the Intima / M+A border is illustrated by yellow lining. **F**: SMC-α positive cells in intimal or M+A layer expressed as a percentage of area occupied. **G:** Number of proliferating SMC-α and Ki-67 double positive cells in the indicated layer per mm^2. **H:** Representative images of neutrophil anti-elastase (brown)-stained vein grafts from LDLr$^{-\backslash-}$ mice preconditioned as indicated and analyzed on post-operative day 4, with the Intima / M+A border depicted in yellow lining. **I:** Quantitation (n=9-10/group) of neutrophil transmigration in intimal layer (805±160 vs. 322±131 cells/mm^2, p=0.0184, Mann Whitney test), PVAT layer (423±73 vs. 220±50 cells/mm^2, p=0.0077, Student's T test) and total vein graft (447±45 vs. 233±61 cells/mm^2 p=0.0146, Student's T test). Scale bars = 100µm as indicated. All data expressed as mean ± SEM; *p<0.05, **p<0.01.

neutrophil transmigration. Thus, reduced intimal hyperplasia observed 28 days after vein grafting in the PR group was associated with a reduced inflammatory response during early vein graft adaptations.

3.3 Contribution of Donor and Recipient Response to Protein Restriction in Attenuation of Vein Graft Disease.

Because thoracic IVC graft donors and recipients are separate mice, we were next able to determine whether the effects of PR were specific to the donor mouse, the recipient or both. Grafts from donors preconditioned on PR and transplanted into recipients on the control diet displayed no statistically significant attenuation of I/M+A area ratio 28 days after surgery (**Fig. 3A**) relative to grafts in which both donor and recipient were fed a control HFHC diet (data from **Fig.1)**. However, grafts from donors fed a control HFHC diet and transplanted into recipients preconditioned on PR displayed significant attenuation of I/M+A area ratio relative to the same control group (p=0.0036, **Fig. 3A**). This effect appeared due in part to an increase in M+A area after preconditioning of the recipient, combined with a tendency of reduced intimal area upon preconditioning of both donor and recipient, but not either alone (**Fig. 3B-C**). Collagen deposition (**Fig. 3D-E)** and luminal diameters (**Fig. 3F**) were not significantly different between diet groups. Taken together, these data suggest that while the major effect of PR on attenuation of vein graft disease is from the recipient, preconditioning of both the donor vein graft and the recipient are necessary to gain a maximal protective response.

3.4 Protein Restriction Upregulates CGL and H_2S Levels in Endothelial Cells.

Dietary preconditioning protects against hepatic ischemia reperfusion injury and has previously been associated with increased expression of the transsufuration enzyme CGL and increased production of the cytoprotective molecule, H_2S.[26] CGL is also the major H_2S-producing enzyme in endothelial cells[36] and is increased in ECs in response to reduced dietary sulfur amino acids.[46] To test the potential role of CGL/H_2S in PR-mediated protection against vein graft disease, we examined localization and expression of CGL as well as CBS, another major H_2S producing enzyme, in vessels. Immunohistochemical analysis of IVCs revealed CBS localization primarily in the medial layer (**Fig. 4A**) and CGL in the endothelium (**Fig. 4B**). Quantitation of protein expression by western blot in extracts of whole thoracic aortas revealed a significant increase in both CGL (p=0.014, **Fig. 4C-D**) and CBS (p=0.021, **Fig. 4C-D**) upon PR-HFHC. However, in ECs isolated from thoracic aortas and IVCs using CD31+ magnetic

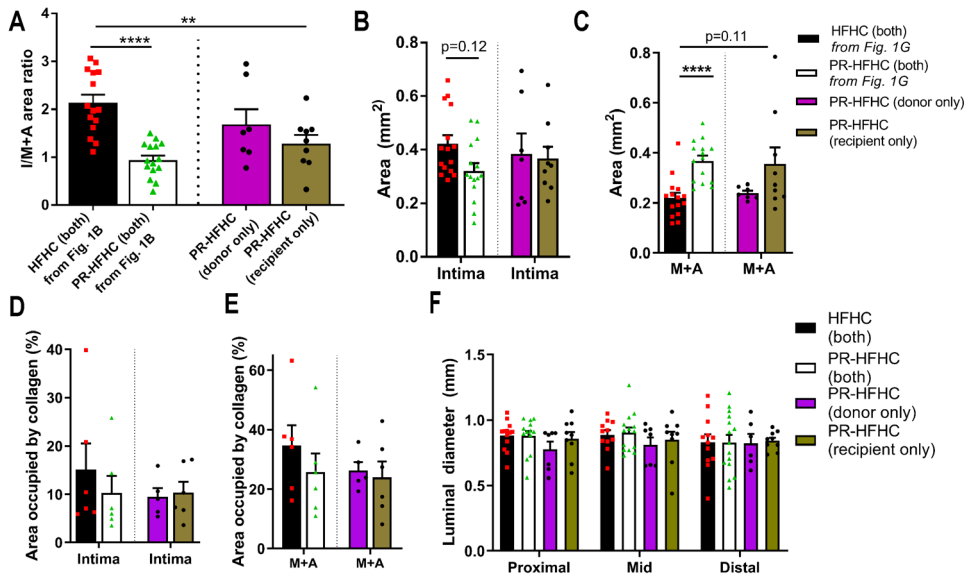

Figure 3. Contribution of Donor and Recipient Response to Protein Restriction in Attenuation of Vein Graft Disease.

A-D: Vein grafts assessments 28 days after surgery in PR-HFHC preconditioned donor only or receipient only vs. neither (HFHC control) or both (PR-HFHC control) from Figure 1. **A:** Intimal(I)/ Media+Adventitia (M+A) area ratios from donors, recipients or both preconditioned on the indicted diet. HFHC (both) and PR-HFHC (both) data are from Fig.1B; PR-HFHC(donor only; 2.1±0.2); PR-HFHC (recipient-only; 1.3±0.2); one-way ANOVA with Dunnett's multiple comparisons test vs. HFHC (both). **B-C:** Intimal **(B)** and M+A**(C)** areas from the indicated diet groups; Kruskal Wallis test with Dunnett's multiple comparisons test vs. HFHC (both). **D-E:** Percent of intimal **(D)** or M+A **(E)** area occupied by collagen; one-way ANOVA. **F:** Vein graft lumen diameter proximal, mid or distal to the heart four weeks after graft implantation as determined by i*n vivo* duplex ultrasound; two-way ANOVA with Turkey's multiple comparisons test. All data expressed as mean ± SEM; **p<0.01, ****p<0.0001.

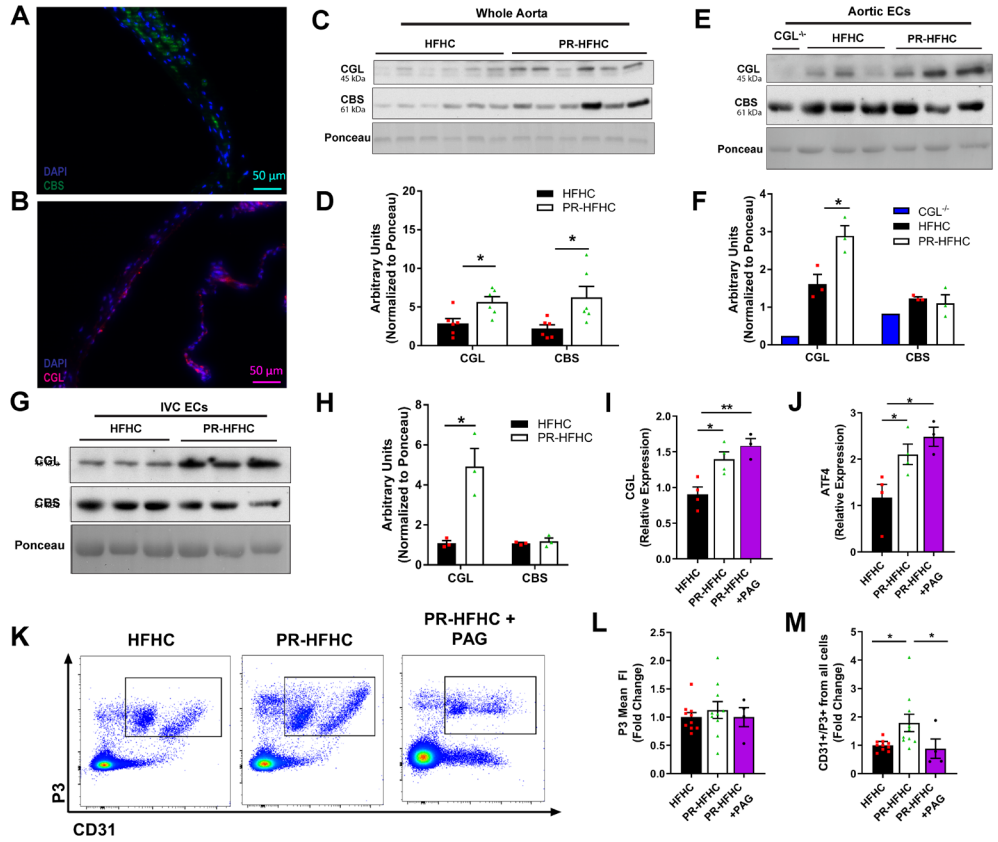

Figure 4. Protein Restriction Upregulates Cystathionine Gamma-Lyase and H_2S Levels in Endothelial Cells.

A,B: Representative images of inferior vena cava (IVC) immunofluorescence staining of CBS **(A)** or CGL **(B)**. **C-H:** Western blots **(C,E,G)** and quantitation **(D,F,H)** of CGL and CBS from whole thoracic aorta (**C-D**), aortic endothelial cells (ECs) **(E-F)** or IVC ECs **(G-H)** isolated from $LDLr^{-\backslash-}$ mice after one week on the indicated diet; Ponceau stained membranes were used as loading controls and $CGL^{-/-}$ ECs were used as a control for CGL antibody specificity **(E)**. **I-M:** $LDLr^{-\backslash-}$ mice in the indicated treatment groups (HFHC, PR-HFHC or PR-HFHC + PAG) were harvested after one week. **I,J**: Whole thoracic aorta gene expression of CGL **(I)** and ATF4 **(J)**; one-way ANOVA with Dunnet's multiple comparisons test vs. HFHC control group. **K-M:** Flow cytometric analysis of single cell isolates from lung after staining with CD31 and P3 (fluorescent H_2S probe). **K**: Representative dot plots from the indicated groups with CD31/P3 double positive cells in the box. **L,M:** Fold change in mean fluorescent intensity of P3 (**L**) and frequency (**M**) of CD31/P3 double positive cells; HFHC & PR-HFHC n=10/group; PR-HFHC+PAG 4/group; Kurskal-Wallis test with Dunnett's multiple comparisons test. All data expressed as mean ± SEM; *p<0.05, **p<0.01.

bead separation, only CGL protein expression was significantly increased upon PR (aorta ECs, p=0.02, **Fig. 4E-F**; IVC ECs**,** p=0.01, **Fig. 4G-H**). Aortic CGL expression was also significantly increased on the mRNA level in the PR group (p=0.015, **Fig. 4I**), as was the transcription factor ATF4 that regulates CGL expression (p=0.044, **Fig. 4J).**

We next measured endogenous H_2S levels in ECs by flow cytometry using a fluorescent H_2S-specific probe.[51] Due to the limited number of ECs in large vessels, ECs were isolated from lungs of LDLr$^{-\backslash-}$ mice after 1 week of control HFHC or PR-HFHC diet feeding. A separate group of PR mice was treated with the CGL inhibitor PAG (daily 20mg/kg IP injection) to inform on CGL-dependent H_2S production. While there was no difference in mean fluorescent P3 intensity (**Fig. 4K-L**) amongst groups, there was an increase in the number of CD31+/P3+ cells in the PR-HFHC group (p=0.031, **Fig 4K, M**) that was prevented by PAG treatment (p=0.04, **Fig. 4K, M**). Interestingly, CGL and ATF4 gene expression remained significantly increased relative to controls in the PAG treatment group, consistent with the post-translational mechanism of PAG action (**Fig. 4I-J**). Taken together, these data suggest that PR significantly increases CGL protein expression and sulfide production specifically in vascular endothelial cells.

3.5 CGL is required for Protein Restriction Mediated Attenuation of Intimal Hyperplasia.

To assess the requirement of CGL activity in the attenuation of IH by PR *in vivo,* donor and recipient LDLr$^{-/-}$ mice injected with either vehicle or PAG during one week of dietary preconditioning (PR + PAG vs. PR + vehicle, 10mg/kg IP) underwent vein graft surgery and postoperative day 28 harvest and assessment. PAG-treated mice showed increased I/M+A area (p=0.014, **Fig. 5A-B**) and thickness ratios (p=0.0144, **Fig. 5C**) with trends toward increased intimal thickness and decreased M+A area and thickness (**Fig. 5D-E**) compared to vehicle-treated controls. These data suggest that CGL is required for the attenuation of intimal hyperplasia by PR during the preconditioning period.

3.6 CGL Overexpression Protects Against Vein Graft Disease but Does Not Increase Basal Endothelial Cell H_2S Levels.

Finally, we asked whether overexpression of CGL could mimic the effects of dietary protein restriction on vein graft disease. For this we engineered a new bacterial artificial chromosome-based CGL transgenic model (CGL^{tg}) containing extra copies of the CGL gene but with endogenous regulatory elements up

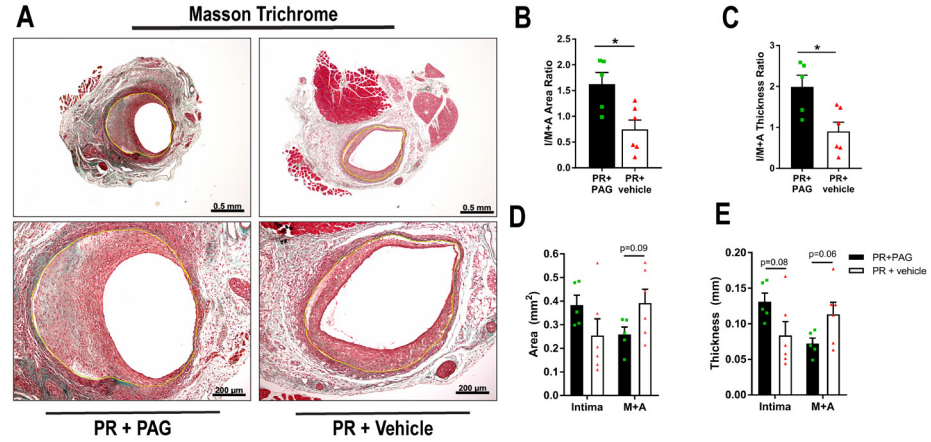

Figure 5. Cystathionine Gamma-Lyase is Required for Protein Restriction Mediated Attenuation of Intimal Hyperplasia.

A-D: Vein grafts assessments 28 days after surgery in PR-HFHC preconditioned LDLr$^{-/-}$ mice injected with either vehicle or PAG during the one week preconditioning period prior to vein grafting; n=5-6/group. **A:** Representative images of Masson trichome-stained vein grafts; boundary between intimal (I) and medial + adventitial (M+A) layers is traced in yellow. Scale bars = 0.5mm & 200µm as indicated. **B,C:** I/M+A area (**B**, 0.7 ± 0.2 vs. 1.6 ± 0.2, p=0.014, Student's T test) and thickness (**C**, 0.9 ± 0.2 vs. 2.0 ± 0.2, p=0.014, Student's T test) ratios. **D,E:** Intimal and M+A area (**D**) and thickness (**E**); Student's T test between ± PAG treatment groups within layer. All data expressed as mean ± SEM; *p<0.05

to 20kb upstream and over 100 kb downstream intact. Increased CGL protein expression (**Fig. 6A-B**) and H_2S production capacity (**Fig. 6C-D**) were confirmed in protein extracts of kidney and liver, respectively, relative to non-transgenic littermate controls. CGL protein was also increased in aortic ECs from CGL^{tg} mice relative to control mice (data not shown).

To test the effects of CGL overexpression on vein graft disease, CGL^{tg} and wild type littermate control mice were placed on a HFHC diet for 3 weeks and then subjected to vein graft surgery. Grafts harvested 28 days after surgery revealed decreased I/M+A area ratio (p=0.0066, **Fig. 6E-F**) and thickness ratio (p=0.0159, **Fig. 6G**) and reduced intimal SMC-actin in CGL^{tg} mice (p=0.032, **Fig. 6J-K**). Duplex biomicroscopy performed during the 4th postoperative week revealed a trend toward increased distal luminal diameter in CGL^{tg} compared to WT mice (**Fig. 6L**) consistent with an outward remodeling phenotype. Furthermore, at post-operative day 4 there was a trend toward reduced intimal neutrophil transmigration in the CGL^{tg} compared to WT mice (**Fig 6M-N**).

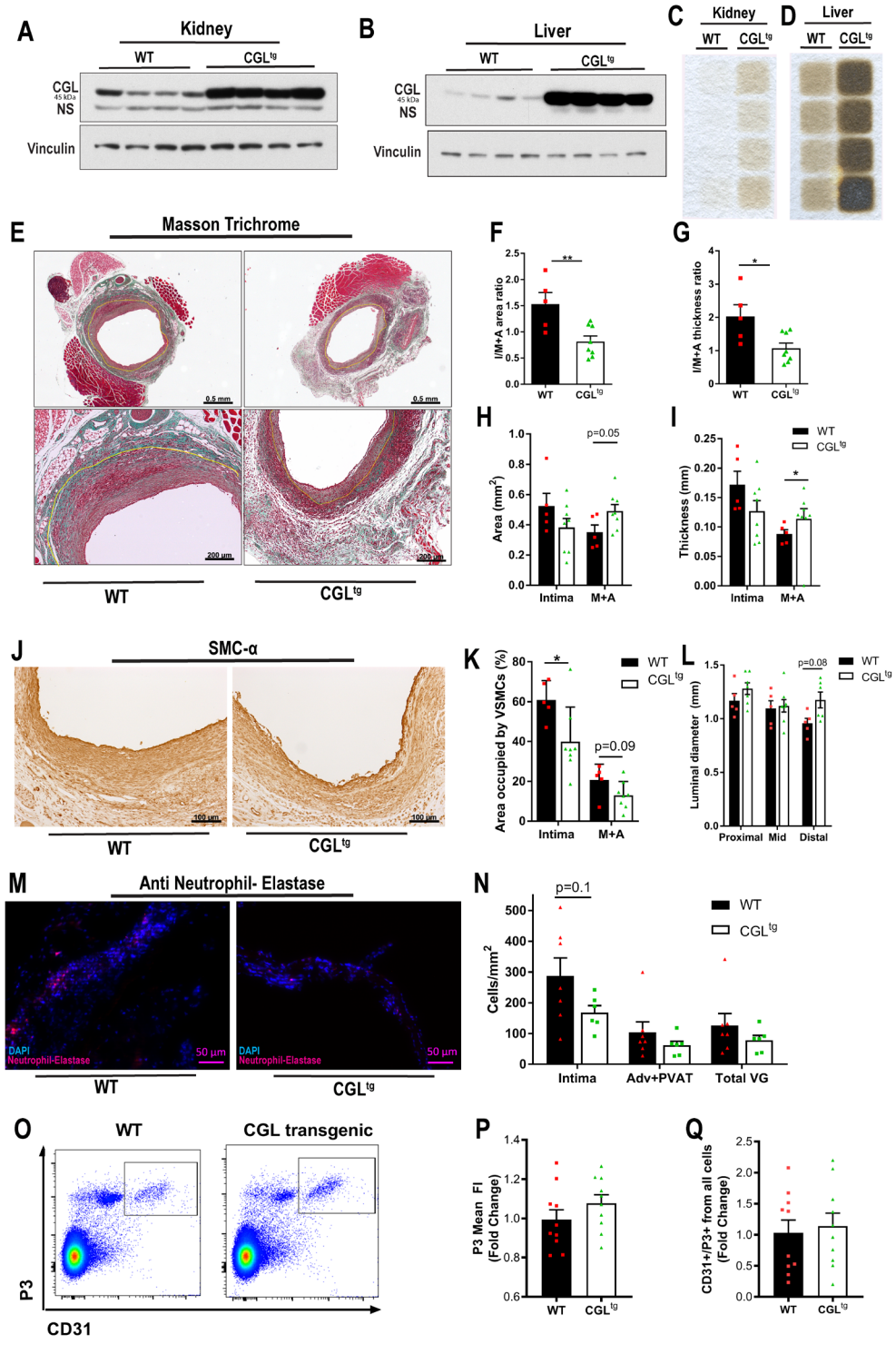

Figure 6. CGL Overexpression Protects Against Vein Graft Disease But Does Not Increase Basal Endothelial Cell H_2S Levels.

A-D: Western blot of CGL (**A-B**) and hydrogen sulfide production capacity (**C-D**) in homogenates of kidney (**A,C**) and liver (**B,D**) from hemizygous CGL transgenic (CGL^{tg}) and WT littermate mice as indicated**. E-N:** Vein grafts assessments in WT vs CGL^{tg} mice (n=5-8/group) 28 days (**E-L**) or 4 days (**M-N**) after surgery. **E**: Representative images of Masson's trichome-stained vein grafts; boundary between intimal (I) and medial + adventitial (M+A) layers is traced in yellow. Scale bars = 0.5mm & 200µm as indicated. **F:** I/M+A area ratios (1.5 ± 0.2 vs. 0.8 ± 0.1, p=0.0066, Student's T test). **G**: I/M+A thickness ratios (2.0 ± 0.4 vs. 1.1 ± 0.2, p=0.0159, Student's T test). **H-I**: Intimal and M+A area (**H**) and thickness (**I**, 0.088±0.007 vs. 0.114±0.017, p=0.0295, Mann Whitney test). **J:** Representative images of vein grafts stained with SMC-α (brown); scale bar = 100µm. **K:** SMC-α positive cells in intimal layer (61 vs. 38, p=0.032, Student's T test) or M+A layer expressed as a percentage of area occupied. **L:** Vein graft lumen diameter proximal, mid or distal to the heart as indicated (two-way ANOVA with Turkey's multiple comparisons test) **M:** Representative images of grafts stained with anti-neutrophil-elastase on post-operative day 4; scale bars = 50µm. **N:** Quantitation of neutrophil transmigration in intimal layer (Student's T test), adventitial + perivascular adiopose tissue (PVAT) layer and total vein graft (Mann-Whitney test). **O-Q**: Endogenous H_2S in lung endothelial cells of WT or CGL^{tg} littermates (n=5/group). **O**: Representative dot plots with CD31/P3 double positive cells indicated within the box. **P, Q**: Fold change in mean fluorescent intensity of P3 (**P**) and frequency (**Q**) of CD31/P3 double positive cells; WT & CGL^{tg} 10/group; Mann-Whitney test. All data expressed as mean ± SEM; *p<0.05, **p<0.01.

Finally, an assessment of endogenous H_2S levels in ECs did not reveal any significant differences in mean fluorescent P3 intensity or the number of CD31+/P3+ cells between genotypes (**Fig. 6O-Q).** Taken together, these data suggest that PR and genetic CGL overexpression both attenuate vein graft disease through partially overlapping mechanisms.

4. Discussion.

Here we tested a dietary intervention involving unlimited access to food in which protein was entirely replaced with carbohydrates for one week, a paradigm that may be more practical for patients than one involving overall food restriction. We found that one week of PR prior to surgery significantly attenuated intimal hyperplasia in a validated mouse vein graft model. Pharmacological inhibition of CGL-dependent H_2S production using PAG abrogated PR-mediated protection from IH, while genetic overexpression using a new BAC-based CGL transgenic overexpressing model revealed protection independent of diet. Reduced neutrophil infiltration in the vein graft on post-

operative day 4 consistent with a reduced proinflammatory response, and reduced smooth muscle cell infiltration into the intima on day 28 signifying an altered fibroproliferative response[11], were common to both diet and genetic models of improved vein graft patency. Neutrophil infiltration on day 4 may occur as a result of local changes in the vessel endothelium, thus linking donor and recipient effects that were both required for maximal protection. A model for the role of CGL in PR-mediated protection against intimal hyperplasia is presented in **Figure 7**.

In support of a potential role of CGL-derived H_2S in vascular injury, it was recently reported that levels of CGL and H_2S from lower extremity muscles were decreased in PAD patients compared to controls [52]. Recent evidence also demonstrates increased DNA methylation of the CGL gene in CAD patients undergoing CABG [53]. In mice, CGL ablation worsens IH [54], possibly due to increased endothelial cell activation[39]. Here, we observed a significant increase in CGL (but not CBS) protein expression upon PR specifically in ECs derived from IVC and aorta, while in whole aorta CBS was also increased and consistent with CBS and CGL localization to the medial and endothelial layers, respectively. Unfortunately, low EC yields from vessels prevented a robust analysis of H_2S levels in these cells by flow cytometry. This was possible in lung-derived EC populations, in which we found an increase in the frequency of P3+ (H_2S-producing) ECs after one week of PR. However, surprisingly, we did not observe a significant an increase in basal H_2S production (P3 mean fluorescence intensity) in either EC or non-EC populations either upon PR or CGL overexpression. These data suggest that changes in H_2S production induced upon surgical stress either in ECs or other non-EC cell population such as neutrophils may be more important to vascular protection than basal levels per se. Future studies in different cell types over a time course after vessel implantation are thus required to investigate whether H_2S production is increased in interventions in which CGL is upregulated. Non-mutually exclusive possibilities include the importance of stored rather than free pools of H_2S, which are currently poorly understood, or the role of CGL in *de novo* cysteine production independent of H_2S generation. Another caveat in the interpretation of these results is the potential functional heterogeneity between vascular beds from a different origin[55], thereby limiting our assessment of sulfide production in vascular conduit beds after PR based on lung ECs.

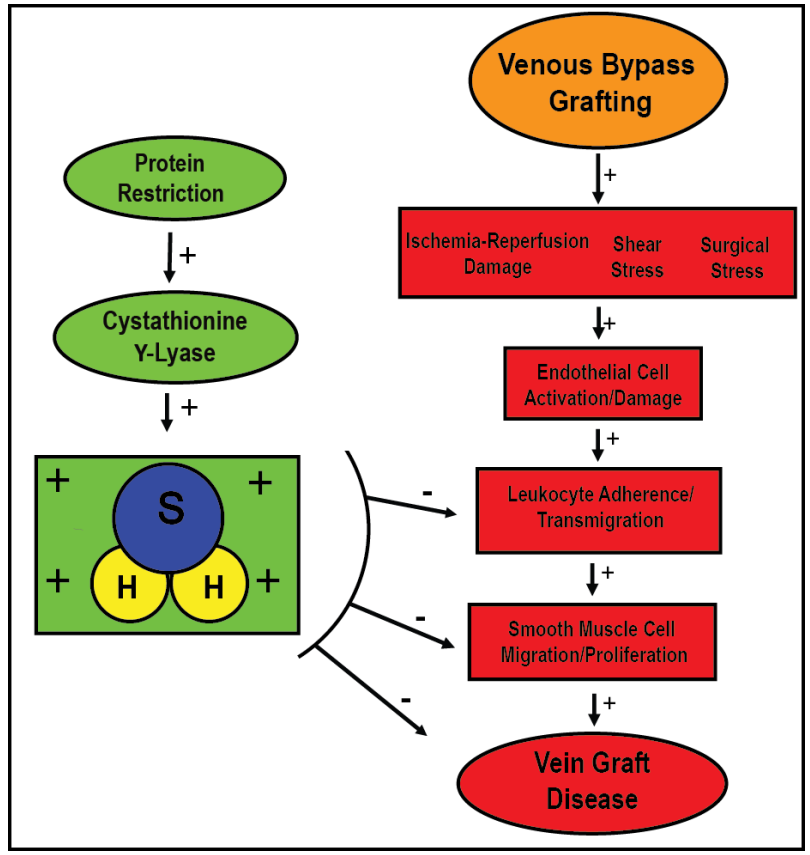

Figure 7. Model for attenuation of vein graft disease by short-term protein restriction via upregulation of cysthathionine-Y-lyase.

While these preclinical data support potential benefits of H_2S manipulation on the vascular response to injury, several limitations are acknowledged. The time course of IH in the mouse vein graft model is short, and the durability of the protection remains unknown. Furthermore, the ability of PR to protect against IH across a range of co-morbidities often encountered in vascular surgery patient (old age, high incidence of diabetes, etc.) were not tested in our murine model. Finally, specific temporal therapeutic windows for upregulation of endogenous H_2S during the peri-operative period for elective procedures remains obscure. Nonetheless, since this strategy may be relevant for other forms of planned vascular injury (endovascular interventions) as well as for organ transplantation, this early work should incite future research to address these limitations.

In conclusion, here we provide foundational rodent data that a simple, translatable dietary intervention attenuates the vascular proliferative response to injury in a clinically relevant model of vein bypass grafting via upregulation of enzymes directly involved in the production of the cytoprotective gaseous transmitter, H_2S.

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KT and PK conceived of experimental designs, performed experiments and wrote the manuscript. MT performed surgeries, collected data, processed and analyzed histology. MM was involved in ex vivo experiments, western blot and data analysis. JHTV analyzed and compensated flow cytometry data. AL was involved in transgenic mouse breeding, husbandry and experimental designs. WT and BNL developed the CGL transgenic mouse model. MRV performed CGL transgenic mouse surgeries and advised with PHQ on data analysis of intimal hyperplasia. JRM and CKO provided funding, experimental designs and mentorship and they oversaw the biologic research protocol and organized the necessary collaborations for project completion. They also worked with the co-authors on manuscript preparation and finalization.

Conflict of Interest: none declared.

7. References.

1. Fontana L, Klein S. Aging, adiposity, and calorie restriction. *JAMA* 2007;**297**:986-994.
2. Welsh JA, Sharma A, Cunningham SA, Vos MB. Consumption of added sugars and indicators of cardiovascular disease risk among US adolescents. *Circulation* 2011;**123**:249-257.
3. Mente A, de Koning L, Shannon HS, Anand SS. A systematic review of the evidence supporting a causal link between dietary factors and coronary heart disease. *Arch Intern Med* 2009;**169**:659-669.
4. Sotos-Prieto M, Bhupathiraju SN, Mattei J, Fung TT, Li Y, Pan A, Willett WC, Rimm EB, Hu FB. Changes in Diet Quality Scores and Risk of Cardiovascular Disease Among US Men and Women. *Circulation* 2015;**132**:2212-2219.
5. Weaver H, Shukla N, Ellinsworth D, Jeremy JY. Oxidative stress and vein graft failure: a focus on NADH oxidase, nitric oxide and eicosanoids. *Current Opinion in Pharmacology* 2012;**12**:160-165.
6. Owens CD, Wake N, Jacot JG, Gerhard-Herman M, Gaccione P, Belkin M, Creager MA, Conte MS. Early biomechanical changes in lower extremity vein grafts--distinct temporal phases of remodeling and wall stiffness. *J Vasc Surg* 2006;**44**:740-746.
7. Osgood MJ, Hocking KM, Voskresensky IV, Li FD, Komalavilas P, Cheung-Flynn J, Brophy CM. Surgical vein graft preparation promotes cellular dysfunction, oxidative stress, and intimal hyperplasia in human saphenous vein. *Journal of Vascular Surgery* 2014;**60**:202-211.
8. Walpola PL, Gotlieb AI, Cybulsky MI, Langille BL. Expression of ICAM-1 and VCAM-1 and monocyte adherence in arteries exposed to altered shear stress. *ArterioscIerThrombVascBiol* 1995;**15**:2-10;Published erratum appears in Arterioscler Thromb Vasc Biol 1995 Mar;1915(1993):1429.
9. Khaleel MS, Dorheim TA, Duryee MJ, Durbin HE, Bussey WD, Garvin RP, Klassen LW, Thiele GM, Anderson DR. High-Pressure Distention of the Saphenous Vein During Preparation Results in Increased Markers of Inflammation: A Potential Mechanism for Graft Failure. *The Annals of Thoracic Surgery* 2012;**93**:552-558.
10. Voisin MB, Nourshargh S. Neutrophil Transmigration: Emergence of an Adhesive Cascade within Venular Walls. *Journal of Innate Immunity* 2013;**5**:336-347.
11. de Vries MR, Simons KH, Jukema JW, Braun J, Quax PHA. Vein graft failure: from pathophysiology to clinical outcomes. *Nat Rev Cardiol* 2016;**13**:451-470.
12. Ambler GK, Twine CP. Graft type for femoro-popliteal bypass surgery. *Cochrane Database Syst Rev* 2018;**2**:CD001487.
13. Conte MS, Bandyk DF, Clowes AW, Moneta GL, Seely L, Lorenz TJ, Namini H, Hamdan AD, Roddy SP, Belkin M, Berceli SA, DeMasi RJ, Samson RH, Berman SS. Results of PREVENT III: A multicenter, randomized trial of edifoligide for the prevention of vein graft failure in lower extremity bypass surgery. *Journal of Vascular Surgery* 2006;**43**:742-751.e741.
14. Song HG, Rumma RT, Ozaki CK, Edelman ER, Chen CS. Vascular Tissue Engineering: Progress, Challenges, and Clinical Promise. *Cell Stem Cell* 2018;**22**:340-354.
15. Almasri J, Adusumalli J, Asi N, Lakis S, Alsawas M, Prokop LJ, Bradbury A, Kolh P, Conte MS, Murad MH. A systematic review and meta-analysis of revascularization outcomes of infrainguinal chronic limb-threatening ischemia. *J Vasc Surg* 2018;**68**:624-633.
16. Bhatt DL. CABG the clear choice for patients with diabetes and multivessel disease. *Lancet* 2018;**391**:913-914.

17. Royse A, Pawanis Z, Canty D, Ou-Young J, Eccleston D, Ajani A, Reid CM, Bellomo R, Royse C. The effect on survival from the use of a saphenous vein graft during coronary bypass surgery: a large cohort study. *Eur J Cardiothorac Surg* 2018.
18. Burnand KM, Lahiri RP, Burr N, Jansen van Rensburg L, Lewis MP. A randomised, single blinded trial, assessing the effect of a two week preoperative very low calorie diet on laparoscopic cholecystectomy in obese patients. *HPB (Oxford)* 2016;**18**:456-461.
19. van Ginhoven TM, de Bruin RW, Timmermans M, Mitchell JR, Hoeijmakers JH, Ijzermans JN. Pre-operative dietary restriction is feasible in live-kidney donors. *Clin Transplant* 2011;**25**:486-494.
20. Fleisher LA, Beckman JA, Brown KA, Calkins H, Chaikof E, Fleischmann KE, Freeman WK, Froehlich JB, Kasper EK, Kersten JR, Riegel B, Robb JF, Acc/Aha Task Force M, Smith SC, Jr., Jacobs AK, Adams CD, Anderson JL, Antman EM, Buller CE, Creager MA, Ettinger SM, Faxon DP, Fuster V, Halperin JL, Hiratzka LF, Hunt SA, Lytle BW, Nishimura R, Ornato JP, Page RL, Riegel B, Tarkington LG, Yancy CW. ACC/AHA 2007 Guidelines on Perioperative Cardiovascular Evaluation and Care for Noncardiac Surgery: Executive Summary: A Report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines (Writing Committee to Revise the 2002 Guidelines on Perioperative Cardiovascular Evaluation for Noncardiac Surgery): Developed in Collaboration With the American Society of Echocardiography, American Society of Nuclear Cardiology, Heart Rhythm Society, Society of Cardiovascular Anesthesiologists, Society for Cardiovascular Angiography and Interventions, Society for Vascular Medicine and Biology, and Society for Vascular Surgery. *Circulation* 2007;**116**:1971-1996.
21. Gallinetti J, Harputlugil E, Mitchell JR. Amino acid sensing in dietary-restriction-mediated longevity: roles of signal-transducing kinases GCN2 and TOR. *Biochem J* 2013;**449**:1-10.
22. Walford RL, Harris SB, Gunion MW. The calorically restricted low-fat nutrient-dense diet in Biosphere 2 significantly lowers blood glucose, total leukocyte count, cholesterol, and blood pressure in humans. *Proc Natl Acad Sci U S A* 1992;**89**:11533-11537.
23. Longchamp A, Harputlugil E, Corpataux JM, Ozaki CK, Mitchell JR. Is Overnight Fasting before Surgery Too Much or Not Enough? How Basic Aging Research Can Guide Preoperative Nutritional Recommendations to Improve Surgical Outcomes: A Mini-Review. *Gerontology* 2017;**63**:228-237.
24. Masoro EJ. Subfield history: caloric restriction, slowing aging, and extending life. *Sci Aging Knowledge Environ* 2003;**2003**:RE2.
25. Mirzaei H, Di Biase S, Longo VD. Dietary Interventions, Cardiovascular Aging, and Disease: Animal Models and Human Studies. *Circ Res* 2016;**118**:1612-1625.
26. Hine C, Harputlugil E, Zhang Y, Ruckenstuhl C, Lee BC, Brace L, Longchamp A, Trevino-Villarreal JH, Mejia P, Ozaki CK, Wang R, Gladyshev VN, Madeo F, Mair WB, Mitchell JR. Endogenous hydrogen sulfide production is essential for dietary restriction benefits. *Cell* 2015;**160**:132-144.
27. Mauro CR, Tao M, Yu P, Trevino-Villerreal JH, Longchamp A, Kristal BS, Ozaki CK, Mitchell JR. Preoperative dietary restriction reduces intimal hyperplasia and protects from ischemia-reperfusion injury. *J Vasc Surg* 2016;**63**:500-509 e501.
28. Harputlugil E, Hine C, Vargas D, Robertson L, Manning BD, Mitchell JR. The TSC complex is required for the benefits of dietary protein restriction on stress resistance in vivo. *Cell Rep* 2014;**8**:1160-1170.

29. Peng W, Robertson L, Gallinetti J, Mejia P, Vose S, Charlip A, Chu T, Mitchell JR. Surgical stress resistance induced by single amino acid deprivation requires Gcn2 in mice. *Sci Transl Med* 2012;**4**:118ra111.
30. Varendi K, Airavaara M, Anttila J, Vose S, Planken A, Saarma M, Mitchell JR, Andressoo JO. Short-term preoperative dietary restriction is neuroprotective in a rat focal stroke model. *PLoS One* 2014;**9**:e93911.
31. Robertson LT, Treviño-Villarreal JH, Mejia P, Grondin Y, Harputlugil E, Hine C, Vargas D, Zheng H, Ozaki CK, Kristal BS, Simpson SJ, Mitchell JR. Protein and Calorie Restriction Contribute Additively to Protection from Renal Ischemia Reperfusion Injury Partly via Leptin Reduction in Male Mice. *The Journal of Nutrition* 2015;**145**:1717-1727.
32. Trocha K, Kip P, MacArthur MR, Mitchell SJ, Longchamp A, Treviño-Villarreal JH, Tao M, Bredella MA, De Amorim Bernstein K, Mitchell JR, Ozaki CK. Preoperative Protein or Methionine Restriction Preserves Wound Healing and Reduces Hyperglycemia. *Journal of Surgical Research* 2019;**235**:216-222.
33. Mitchell JR, Verweij M, Brand K, van de Ven M, Goemaere N, van den Engel S, Chu T, Forrer F, Muller C, de Jong M, van IW, JN IJ, Hoeijmakers JH, de Bruin RW. Short-term dietary restriction and fasting precondition against ischemia reperfusion injury in mice. *Aging Cell* 2010;**9**:40-53.
34. Mitchell JR, Beckman JA, Nguyen LL, Ozaki CK. Reducing elective vascular surgery perioperative risk with brief preoperative dietary restriction. *Surgery* 2013;**153**:594-598.
35. Mejia P, Trevino-Villarreal JH, Hine C, Harputlugil E, Lang S, Calay E, Rogers R, Wirth D, Duraisingh MT, Mitchell JR. Dietary restriction protects against experimental cerebral malaria via leptin modulation and T-cell mTORC1 suppression. *Nat Commun* 2015;**6**:6050.
36. Yang G, Wu L, Jiang B, Yang W, Qi J, Cao K, Meng Q, Mustafa AK, Mu W, Zhang S, Snyder SH, Wang R. H2S as a physiologic vasorelaxant: hypertension in mice with deletion of cystathionine gamma-lyase. *Science* 2008;**322**:587-590.
37. Wallace JL, Blackler RW, Chan MV, Da Silva GJ, Elsheikh W, Flannigan KL, Gamaniek I, Manko A, Wang L, Motta JP, Buret AG. Anti-inflammatory and cytoprotective actions of hydrogen sulfide: translation to therapeutics. *Antioxid Redox Signal* 2015;**22**:398-410.
38. Wang R. Physiological Implications of Hydrogen Sulfide: A Whiff Exploration That Blossomed. *Physiological Reviews* 2012;**92**:791-896.
39. Bibli SI, Hu J, Sigala F, Wittig I, Heidler J, Zukunft S, Tsilimigras DI, Randriamboavonjy V, Wittig J, Kojonazarov B, Schurmann C, Siragusa M, Siuda D, Luck B, Abdel Malik R, Filis KA, Zografos G, Chen C, Wang DW, Pfeilschifter J, Brandes RP, Szabo C, Papapetropoulos A, Fleming I. Cystathionine gamma Lyase Sulfhydrates the RNA Binding Protein HuR to Preserve Endothelial Cell Function and Delay Atherogenesis. *Circulation* 2018.
40. Papapetropoulos A, Pyriochou A, Altaany Z, Yang G, Marazioti A, Zhou Z, Jeschke MG, Branski LK, Herndon DN, Wang R, Szabo C. Hydrogen sulfide is an endogenous stimulator of angiogenesis. *Proc Natl Acad Sci U S A* 2009;**106**:21972-21977.
41. King AL, Polhemus DJ, Bhushan S, Otsuka H, Kondo K, Nicholson CK, Bradley JM, Islam KN, Calvert JW, Tao YX, Dugas TR, Kelley EE, Elrod JW, Huang PL, Wang R, Lefer DJ. Hydrogen sulfide cytoprotective signaling is endothelial nitric oxide synthase-nitric oxide dependent. *Proc Natl Acad Sci U S A* 2014;**111**:3182-3187.
42. Zanardo RCO, Brancaleone V, Distrutti E, Fiorucci S, Cirino G, Wallace JL. Hydrogen sulfide is an endogenous modulator of leukocyte-mediated inflammation. *The FASEB Journal* 2006;**20**:2118-2120.

43. Greaney JL, Kutz JL, Shank SW, Jandu S, Santhanam L, Alexander LM. Impaired Hydrogen Sulfide-Mediated Vasodilation Contributes to Microvascular Endothelial Dysfunction in Hypertensive Adults. *Hypertension* 2017;**69**:902-909.
44. Shuang T, Fu M, Yang G, Wu L, Wang R. The interaction of IGF-1/IGF-1R and hydrogen sulfide on the proliferation of mouse primary vascular smooth muscle cells. *Biochem Pharmacol* 2018;**149**:143-152.
45. Robertson LT, Trevino-Villarreal JH, Mejia P, Grondin Y, Harputlugil E, Hine C, Vargas D, Zheng H, Ozaki CK, Kristal BS, Simpson SJ, Mitchell JR. Protein and Calorie Restriction Contribute Additively to Protection from Renal Ischemia Reperfusion Injury Partly via Leptin Reduction in Male Mice. *J Nutr* 2015;**145**:1717-1727.
46. Longchamp A, Mirabella T, Arduini A, MacArthur MR, Das A, Trevino-Villarreal JH, Hine C, Ben-Sahra I, Knudsen NH, Brace LE, Reynolds J, Mejia P, Tao M, Sharma G, Wang R, Corpataux JM, Haefliger JA, Ahn KH, Lee CH, Manning BD, Sinclair DA, Chen CS, Ozaki CK, Mitchell JR. Amino Acid Restriction Triggers Angiogenesis via GCN2/ATF4 Regulation of VEGF and H2S Production. *Cell* 2018;**173**:117-129 e114.
47. Yu P, Nguyen BT, Tao M, Campagna C, Ozaki CK. Rationale and practical techniques for mouse models of early vein graft adaptations. *J Vasc Surg* 2010;**52**:444-452.
48. Jungblut M, Oeltze K, Zehnter I, Hasselmann D, Bosio A. Standardized preparation of single-cell suspensions from mouse lung tissue using the gentleMACS Dissociator. *J Vis Exp* 2009.
49. Hine C, Mitchell JR. Endpoint or Kinetic Measurement of Hydrogen Sulfide Production Capacity in Tissue Extracts. *Bio Protoc* 2017;**7**.
50. de Vries MR, Quax PHA. Inflammation in Vein Graft Disease. *Front Cardiovasc Med* 2018;**5**:3.
51. Singha S, Kim D, Moon H, Wang T, Kim KH, Shin YH, Jung J, Seo E, Lee SJ, Ahn KH. Toward a selective, sensitive, fast-responsive, and biocompatible two-photon probe for hydrogen sulfide in live cells. *Anal Chem* 2015;**87**:1188-1195.
52. Islam KN, Polhemus DJ, Donnarumma E, Brewster LP, Lefer DJ. Hydrogen Sulfide Levels and Nuclear Factor-Erythroid 2-Related Factor 2 (NRF2) Activity Are Attenuated in the Setting of Critical Limb Ischemia (CLI). *J Am Heart Assoc* 2015;**4**.
53. Giannakopoulou E, Konstantinou F, Ragia G, Tavridou A, Karaglani M, Chatzaki E, Papapetropoulos A, Mikroulis D, Manolopoulos VG. Epigenetics-by-Sex Interaction for Coronary Artery Disease Risk Conferred by the Cystathionine gamma-Lyase Gene Promoter Methylation. *OMICS* 2017;**21**:741-748.
54. Yang G, Li H, Tang G, Wu L, Zhao K, Cao Q, Xu C, Wang R. Increased neointimal formation in cystathionine gamma-lyase deficient mice: role of hydrogen sulfide in alpha5beta1-integrin and matrix metalloproteinase-2 expression in smooth muscle cells. *J Mol Cell Cardiol* 2012;**52**:677-688.
55. Potente M, Makinen T. Vascular heterogeneity and specialization in development and disease. *Nat Rev Mol Cell Biol* 2017;**18**:477-494.