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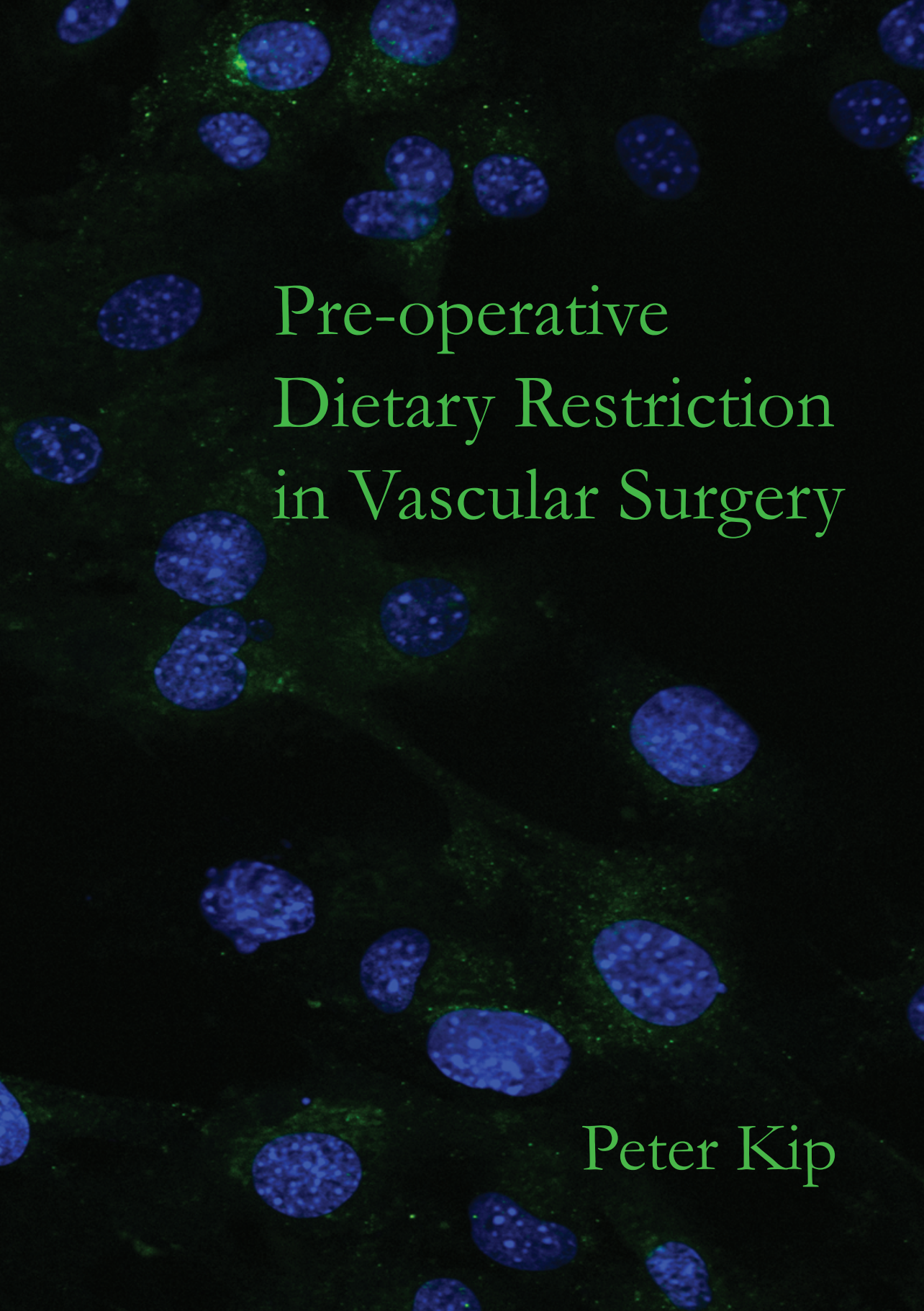
Short-term pre-operative dietary restriction in vascular surgery

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Pre-operative Dietary Restriction in Vascular Surgery

Peter Kip

Short-term Pre-operative Dietary Restriction in Vascular Surgery

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Short-term Pre-operative Dietary Restriction in Vascular Surgery

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This thesis is dedicated to the memory of

Professor James R. Mitchell
1971-2020

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Chapter 1.

General Introduction and Thesis Outline.

Atherosclerotic Occlusive Disease.

Globally, over 200 million individuals suffer from peripheral artery disease (PAD)[1] which is frequently accompanied by coronary artery disease (CAD).[2, 3] Both CAD and PAD are occlusive arterial diseases that can lead to (partial) obstruction of blood flow, which results in local or downstream hypoxic tissue.[4] The pathophysiology of PAD and CAD is mainly driven by atherosclerosis[5], which is a multifactorial inflammatory lipid loading disease of the vascular wall.[6] Atherosclerosis is a chronic and progressive disease, and is characterized by the formation of a lipid rich core in the intimal layer of the vascular wall. This initial process is triggered by elevated low-density lipoprotein cholesterol[7], and exacerbated by risk factors such as obesity, smoking, lack of exercise and hypertension.[8] After this initial lipid-rich core is established in the vascular wall, it will then progress towards a mid-stage atherosclerotic plaque that is characterized by both a necrotic core and uncontrolled inflammation.[9] If plaque growth continues, it can eventually lead to partial obstruction (i.e. stenosis) or a complete impairment of blood flow (i.e. occlusion). More frequently, the plaque can become unstable and eventually erode or rupture. Both plaque stenosis/occlusion and erosion/rupture can lead to local and downstream ischemic tissue[10] and its clinical manifestation is dictated by the anatomical location of the diseased artery. Occurring in a coronary artery, it will give rise to a myocardial infarct and subsequent heart failure.[4] In arteries of the lower extremity, i.e. PAD lesions, the disease commonly manifests as lower extremity pain (i.e. intermittent claudication) or (chronic end-stage or acute) critical limb ischemia in severe cases.[11]

(Cardio)vascular Surgery Interventions.

Lifestyle interventions, statins and anti-platelet drugs are employed to address risk factors for the development of PAD/CAD, consequently slowing the progression of atherosclerotic lesions.[12] And although these are effective in delaying disease onset and progression, unfortunately currently no drugs exist that can mitigate severe clinically manifest coronary/peripheral atherosclerotic occlusive disease.[13] In patients presenting with an acute occlusion of a coronary artery, i.e. acute coronary syndrome/myocardial infarct, percutaneous coronary intervention (PCI) with stent (bare-metal or drug-eluting) is the gold standard.[14] In sub-acute CAD, patients will receive either PCI or coronary artery bypass grafting (CABG). Open bypass surgery is preferred generally when multi-vessel or extensive left main disease is present[15, 16], while an endovascular approach is the treatment of choice in case of single vessel disease and/or a healthy subject.[15, 17] In CABG, the internal mammary artery (IMA) is the preferred conduit compared to other possible arterial and venous grafts, with excellent results.[18, 19] However, especially in multi-vessel CAD, utilization of the greater saphenous vein can be necessary to bypass the occluded arteries.[19]

In vascular surgery practice, intermittent claudication (i.e. symptomatic PAD) is initially treated non-invasively via lifestyle interventions[20], anti-platelet drug therapy[21] and supervised exercise training.[11] In case of severe claudication or critical limb ischemia (chronic or acute), open or endovascular revascularization is necessary to restore blood flow and achieve limb salvage.[22-24] Depending on the location and size of the atherosclerotic plaque, the intervention can either be performed endovascular, open or via a hybrid combination of both.[11, 20, 24] The occlusion/stenosis can be graded according to the TASC classification system[25, 26], in which focal stenosis or occlusions over short lengths are graded TASC A-B and deemed suitable for an endovascular approach. Multiple or recurrent stenosis/occlusions over longer lengths of artery are graded TASC C- D and are candidates for open revascularization via either endarterectomy, bypass surgery or a combination.[26, 27] Endovascular interventions include balloon angioplasty, drug-coated balloons, bare-metal or drug-eluting stents, or endovascular atherectomy. While open interventions include simple endarterectomy, endarterectomy with a patch or bypass surgery employing either venous or prosthetic grafts.[20, 24, 28]

(Cardio)vascular Surgery Interventions Have High Complication and Failure Rates.

Surgical Interventions in Coronary Artery Disease.

Amongst open and endovascular cardiac interventions, PCI is commonly performed and preferred in single vessel disease.[29] Outcome and success rates depend on the type of lesion, patient population and choice of stent (drug-eluting, bare-metal). On average, stent restenosis will occur in between 4.1 - 11% of patients at one-year post-PCI.[30-34] In multi-vessel and left main coronary artery disease however, CABG results in lower 5-year all-cause mortality compared to PCI.[15] In bypass surgery, success rates will mainly depend on the type and the quality of the conduit used to bypass the artery. Graft durability after a bypass with bilateral or lateral IMA is excellent, with patency rates at around 90% 10 years post-operatively.[35, 36] When the IMA is not suited for grafting, or not of sufficient length, the greater saphenous vein is used to create a saphenous vein graft (SVG) and indeed is used more frequently in clinical practice.[37] At 1-year post-intervention, SVG patency rates are at 75%[38] while at 10-15 years post-surgery this further falls toward 50-60%.[39, 40]

Surgical interventions in Peripheral Artery Disease.

In lower extremity vascular surgery few trials have been completed that directly compared open bypass surgery with endovascular procedures in case of (chronic) limb- threatening ischemia. The BASIL trial compared bypass surgery with balloon angioplasty and found higher 2-year survival after an open bypass surgery. In that same study, one-year reintervention rates for bypass surgery and endovascular interventions were 18% and 26% respectively.[41] Since the BASIL trial was hindered by low power and a single endovascular approach (balloon angioplasty), more research is needed. Currently, several trials are underway to address this gap in knowledge regarding effectiveness and durability differences between these two approaches, including the BEST-CLI trial.[42-44] For the treatment of intermittent claudication of the upper leg, endovascular surgery is widely used and in case of short lesion-length (<15cm) with excellent results: one-year primary patency ranges between 67-85%.[45-47] For longer- and femoropopliteal obstructions, endovascular approaches may not be possible and an open bypass is warranted. Saphenous vein bypasses have a 5-year primary patency between 65-75%[48-50], while one large randomized controlled trial found a one-year primary patency as low as 61%.[51] For prosthetic conduits, patency rates are even lower and vary between 27-37%.[50, 52] Intervention failure-

rates notwithstanding, procedure success is also hampered by high incidence of peri-[53] and post-operative cardiovascular events (myocardial infarct, stroke and death)[54] and frequent poor wound healing.[55]

(Cardio)vascular Interventions Fail due to Vascular Remodeling and Vein Graft Disease.

In both open (vein graft) surgery and endovascular interventions alike, surgical injury and ischemia-reperfusion injury to the vessel wall is a common denominator of all procedures. For endovascular approaches this results in mechanical and oxidative damage to endothelial cells (EC) lining the artery, whether via stent placement or balloon angioplasty.[13, 56] In open bypass surgery, in addition to **ischemia-reperfusion damage** and **mechanical/surgical stress**, the vein is also subjected to **altered hemodynamic forces** during and after a successful procedure.[13, 57]

Early Failure and Mid-term Failure after Revascularization.

Broadly speaking, intervention failure can be divided in three temporal windows with a distinct underlying pathophysiology; early, mid- and late-term failure. Early failure, which occurs days to months after the procedure, can mostly be attributed to acute thrombosis after surgical damage to the endothelial lining, both in arteries/stents[58] and vein grafts.[57, 59] In coronary vein grafts for example, generally thrombosed vein grafts make up between 10-25% of all vein grafts that eventually fail at one-year post-operatively.[40, 60]

Mid-term failure after revascularization is generally due to **intimal hyperplasia** (IH) of the vein graft[61, 62] or artery[63-65], and is characterized by uncontrolled migration and proliferation of **vascular smooth muscle cells** (VSMC). VSMCs migrate from the adventitial and medial layers of the artery/vein into the intimal layer, ultimately restricting or occluding blood flow.[66-68] This pathological myo-fibroproliferative response is already triggered during surgery, where depending on the procedure (arterial or venous) several processes impact the vascular wall simultaneously. In vein graft surgery, greater saphenous vein harvesting activates the luminal ECs and medial/adventitial VSMCs of the conduit.[69, 70] As for arterial (endovascular) procedures the EC lining is damaged and/or activated, due to the mechanical/surgical forces exerted on the vascular wall.[71, 72] Besides this mechanical/surgical aspect, other causatives include ischemia-reperfusion injury[68] and oxidative stress.[56, 73] Specifically in bypass surgery, transplantation of the vein from a low-flow and pressure system into an arterial circulation culminates in altered flow and an increase in shear stress and circumferential diameter.[74] This sudden shift in **hemodynamic forces** on the venous wall further contributes towards EC dysfunction in vein grafts.[75]

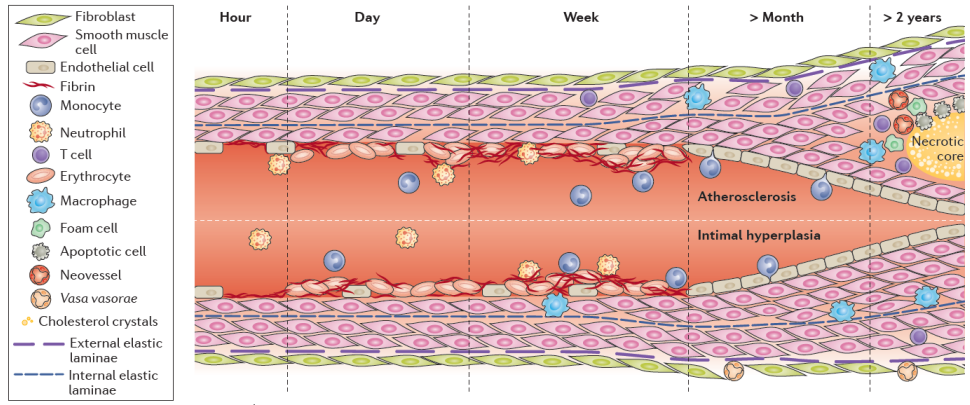


Figure 1. Mechanisms of mid- and late-term revascularization failure. *From: de Vries et al.* Nature Cardiology Reviews. 2016

Extensive surgical stress, local milieu and hemodynamic changes all lead to **dysfunctional endothelium** which upregulates various receptors, such as intracellular adhesive molecules[76] and selectins.[77] These receptors in turn facilitate the homing, attachment and subsequent **transmigration of circulating leukocytes,** while also enabling platelet attachment.[68, 70, 77, 78] Damaged endothelium is not only prone to increased leukocyte attachment and transmigration, but also enables direct inflammatory cell transmigration via dysfunctional inter-EC tight junctions.[77] What is more, the extracellular matrix (ECM) on the basal side of luminal ECs will activate and damage after ischemia-reperfusion injury and stretching of the vessel wall.[79-81] Together with damaged ECs and infiltrated leukocytes, these in turn release damage-associated-molecular patterns (DAMPs)[82] and other pro-inflammatory signaling molecules such as cytokines and chemokines. Upon the release of these molecules, both the local and systemic innate immune response will be activated.[83] Together with the surgical injury itself and subsequent ischemia-reperfusion injury, this will facilitate and enhance the local influx of circulating innate immune cells. These innate cells, which mostly include neutrophils and monocytes, will in turn activate their residential counter-parts as well as other cell types and subsets.[83-85]

This pro-**inflammatory** and pro-remodeling response to vascular injury is not only prompted via perturbations that originate from the lumen and traverse into the vascular wall, but can also stem from adventitial and perivascular tissue layers.[86-88] Local adventitial fibroblast and leukocyte subsets can activate and enhance the intimal hyperplastic signaling cascade (VSMC migration and proliferation), via paracrine signaling in response to mechanical stress and systemic inflammation.[81, 89, 90] Furthermore, most veins and arteries are enveloped by **perivascular adipose tissue (PVAT)**[91], a paracrine organ that not only maintains vascular tone and function but can also initiate and facilitate metabolic and inflammatory signaling pathways.[92] PVAT can be grouped in adipocytes and an interspersed stromal vascular fraction; which consists of residential leukocyte subsets, ECs and VSMCs. Local blood supply to the PVAT and adventitia is facilitated by the latter two cell types of this stromal vascular fraction.[91] Healthy PVAT function can be undermined by various acute and chronic stressors. For example, both obesity[93, 94] and endovascular surgical injury to the underlying blood vessel[95] will alter PVAT phenotype. **Dysfunctional PVAT** in turn will have a distinct pro-inflammatory[96, 97] and pro-atherosclerotic[98, 99] impact on the vascular wall. This "sick fat" can accelerate the intimal hyperplastic response[100] via increased oxidative stress[88, 101], and production of pro-inflammatory adipokines and cytokines.[102, 103]

The aforementioned impact of surgical and hemodynamic stress on dysfunctional ECs and ECM, followed by leukocyte transmigration, adventitial fibroblast signaling, PVAT and residential immune-cell activation; all contribute towards induction of VSMC proliferation and migration.[104] VSMC activation can occur via cell-cell, cytokine-cell and adipokine-cell interactions; but also in response to chemo-attractants.[105] Taken together these stimuli will **trigger VSMCs to undergo phenotypic switching** from a contractile towards a secretory state, which enables them to migrate to- and proliferate in the intimal layer of the vascular wall.[106] This process of medial/adventitial IH is largely responsible for mid-term failure of grafts and arteries alike[13], but if VSMC growth continues and lesion size increases eventually a atherosclerotic plaque can form.[13, 57]

Late-term Failure after Revascularization.

Post-interventional formation of atherosclerotic plaques can occur in up to 30% of arteries after an endovascular intervention[107], with similar findings in coronary vein grafts 3-years post-implantation.[108] Hypo-oxygenation of the growing VSMC lesion, accompanied by chronic inflammation and foam cell formation will contribute to the **formation of a necrotic core**.[10] This state of hypoxia and malnutrition within the intimal layer will trigger **neo-vessels** to grow from the adventitial layer inward and supply the lesion with oxygenated blood and nutrients. These vessels in turn are prone to leakage of erythrocytes which will further destabilize the lesion.[9] Eventually this atherosclerotic plaque will **either occlude the lumen, rupture or erode**, culminating in late-term revascularization failure and return of PAD/CAD symptoms.[9, 13, 109]

No Therapies Exist to Improve Success rates after Revascularization.

High peri-operative-[53, 54] and wound complication rates,[110] long in-hospital stays[111] and frequent reintervention[112, 113]; taken together highly impacts both patients, their family and the healthcare system. Despite decades of research, currently no therapies are available to counter mid- and late-term revascularization failure, whether in vein grafts[57, 114] or arteries[13], nor do strategies exist to limit peri-operative and post-operative complication risks for these patients. **In this thesis the effects of various forms of dietary restriction will be discussed as strategies to limit complication risks.**

Long-term Dietary Restriction as a Strategy to Slow Aging and other Chronic Diseases.

Over a century ago, Osborne and colleagues in 1917 subjected rats to limited access of food during the first 17 months after birth. In their follow-up analysis they found that these food-restricted rats had an extended life-span compared to their counterparts who had been on an a*d libitum* (AL) diet all their life.[115] Then in 1935 McCay and colleagues were able to repeat this finding in a significantly larger cohort of rats, and showed that long-term caloric restriction, i.e. limiting the total daily food intake (CR), was able to prolong lifespan in rats.[116] Four years earlier, in 1931, Slonaker et al. found a similar effect on the extension of life-span in rats by long-term restricting their protein intake (PR) while maintaining caloric intake (i.e. isocaloric).[117]

Subsequent research in a wide range of organisms, from worms[118] to nonhuman primates[119], built on these initial findings and established the effectiveness of long-term CR on extending lifespan, promoting healthy aging and decreasing cardiovascular disease risk.[120] And for PR, rodent data shows similar results in lifespan and metabolic studies.[121, 122] Interestingly, as an alternative to diminished intake of total calories or proteins, restriction of single amino acids in the diet can yield similar benefits. Long-term tryptophan restriction[123] but also restriction of the sulfur amino acids methionine and cysteine (MetR)[124],

again extends lifespan in rats. All these approaches to restrict certain aspect of regular dietary intake, whether this pertains total calories, total proteins, single amino acids or a combination, can be categorized as dietary restriction (DR) and appear to share common pathways towards their favorable phenotype.

Dietary Restriction Confers Benefits Beyond Lifespan Extension.
Beyond aging research, studies found that DR can lower blood pressure and circulating lipids while improving glucose homeostasis and insulin sensitivity.[125, 126] These findings indicate the potential of DR to mitigate several chronic stressors besides aging, by undercutting risk factors for cardiovascular disease and improvement of metabolic health and fitness.[120, 127-129] Interestingly, besides these chronic conditions, long-term DR is also effective in mitigating acute stressors such as ischemia-reperfusion injury to the brain[130] and heart.[131]

In those studies, however, as for most long-term DR interventions, rodents were subjected to DR (30-40% CR in both studies) from an early age. Long-term CR in humans did show improvement regarding cardiovascular risk factors, but also highlighted compliance issues in adhering to this dietary regimen.[132] It can be deducted therefore, that the potential benefits of these diets, that require restriction of calories/proteins for months to years and often from an early age onward, are not compatible with a setting of planned acute and transient stressors such as elective surgery.

Short-term Dietary Restriction and the Host Response to Surgical Injury.

It was Mitchell and colleagues[133] who in 2010 modulated this concept of long-term DR towards a brief period of preconditioning before undergoing surgery. In that seminal work, they subjected mice to either four weeks of 30% CR, a 3-day fast or an *Ad Libitum (*AL) diet and performed renal ischemia-reperfusion injury surgery. During this procedure the renal artery is clamped bilaterally for 30-35 minutes, followed by clamp-removal and subsequent reperfusion. Then immediately post-op all mice are switched back to the AL diet. About 60% of AL-fed mice died in the days following the procedure, while all animals in both the 30% CR and 3-day fasting cohorts survived the surgery. Next to absence of mortality, both diet groups were also protected from post-operative kidney dysfunction, as seen by decreased circulating levels of creatinine and urea compared to the AL group. In that same study, mice were subjected to 1-3 days of fasting followed by surgical hepatic ischemia-reperfusion. Both 1- and 3-day water only fasting groups had significantly lower serum markers of liver damage (ALAT) compared to AL mice.[133] In this first study, the authors established that DR can have rapid onset benefits, and that such short-term restriction of calories is sufficient to protect from acute stress such as renal/ hepatic IRI surgery.

Restriction of Total Proteins or Specific Amino-Acids Also Protects from Surgical Stress.
Although a short-term reduction in calories to achieve surgical stress resistance is favorable compared to any long-term CR diet, a reduction in food intake can be difficult to adhere to regardless the duration of the dietary intervention. In a follow-up study, mice were fed an isocaloric PR diet (proteins were replaced with carbohydrates while maintaining caloric intake), a diet lacking only tryptophan, or a complete diet before undergoing renal or hepatic IRI.[134] A 6-day restriction of just proteins yielded a similar protective phenotype compared to a 3-day water only fast. What is more, mice on a tryptophan restricted complete diet were equally protected from renal IRI.

Subsequent analysis showed that these effects of tryptophan restriction proved dependent on the amino acid deprivation sensor general control non depressible 2 (GCN2).[135] GNC2 in turn is upstream of a broader integrated stress response pathway (ISR) present in all mammalian cells.[136] It was previously reported that in response to a long-term MetR diet, GNC2 phosphorylates eukaryotic transcription factor–2 (eLF2) followed by activation of activating transcription factor-4 (ATF4) which then coordinates downstream metabolic signaling.[137]

Short-term Dietary Restriction Extends Protection Beyond Surgical Injury.
Next the hypothesis that short-term DR can mitigate various forms of acute stress was expanded beyond acute surgical stress. In a model of focal stroke injury, to mimic perioperative stroke, rats were subjected to 6 days of preconditioning with PR. Compared to their counterparts on a control-fed diet, PR-fed rats had limited ischemic injury and accelerated functional recovery.[138] In a preclinical model of cerebral malaria infection, mice where started on DR (40% CR) within 2 days of *P. Berghei* infection. This in turn afforded protection from neurological complications while parallelly significantly improving survival. In this study, short-term DR suppressed the leptin – mTORC1 (mechanistic target of rapamycin complex 1) – axis, which resulted in limited splenic CD8+ T-cell activation and subsequent attenuation of late-stage malaria induced neuropathology.[139]

Combining Caloric and Protein Restriction Results in Additive Protection from Surgical Stress.

Although CR and PR appear to share pathways that result in comparable phenotypes after surgical injury, it remained undetermined whether both were equally effective. Interestingly, after a side-by-side comparison it was revealed that protection was best afforded by combining a reduction in calories with a reduction in proteins. This strategy, i.e. protein-calorie restriction (PCR), was able to reduce surgical stress more effectively after renal IRI compared to either of the singular dietary interventions.140 An initial study in vascular surgery models asked the question whether diet can influence the intimal hyperplastic response to vascular injury and was performed in a focal stenosis model of the right common carotid artery (RCCA). In this model, a 9/0 suture is placed around the RCCA via which blood flow is then partially obstructed.141 Mice were first subjected to diet-induced obesity before vascular wall injury was created, which exacerbated the remodeling response compared to their lean counterparts.[142] Interestingly, in a follow-up study mice were first fed a high-fat diet and then switched to PCR before undergoing surgery. This dietary switch proved capable of decreasing intimal area along the arterial wall proximal from the stenosis, while the ratio between intima and media was not different, suggestive of a DR-induced limitation of the IH response rather the favorable overall remodeling.[143]

Short-term Dietary Restriction and Endogenous Hydrogen Sulfide Upregulation.

Hine et al. in 2015 revealed that increased production of **endogenous hydrogen sulfide (H_2S)** is an essential underlying mechanism of DR-mediated protection.[144] In their study, they supplemented mice subjected to DR with the sulfur amino acids methionine and cysteine, and this in turn failed to protect from hepatic IRI. Consequently, they found that sulfur amino acid restriction (which is part of both CR and PR) activates the transsulfuration pathway (TSP), which produces H_2S as a metabolite. This TSP pathway is an evolutionary conserved pathway consisting of several amino acids sensing enzymes, including cystathionine β-synthase (CBS), cystathionine y-lyase (**CGL**) and 3-mercaptopyruvat sulfur transferase (MST). All three can produce H_2S, but within the vasculature H_2S is mainly derived from CGL.[145]

At physiological levels, H_2S can regulate blood pressure and maintain vascular tone and function within the cardiovasculature.[146] These acute effects of H_2S, which include vasorelaxation/dilation and regulation of vessel-wall permeability, are triggered via activation or inhibition of various ion-channels

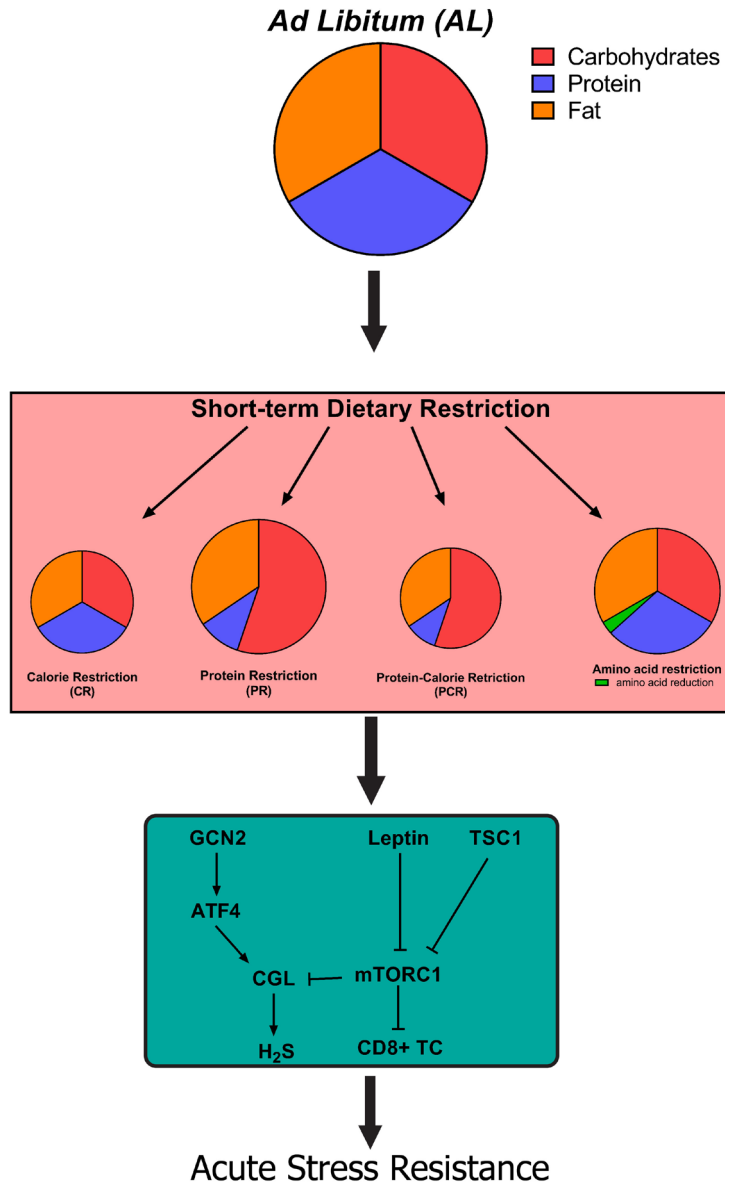


Figure 2. Types of Dietary Restriction and known mechanisms via which dietary restriction improves acute stress resistance. Size of diet charts indicates total calories.

and kinases/enzymes respectively. Vasodilation is prompted via K^+ and Ca^{2+}-channel activation, while vessel-wall permeability is increased via activation of Claudin-5 and VE-cadherin.[147] Next to this physiological role, reduced levels of H_2S have been implicated in numerous diseases and pathophysiological processess.[148] Several studies with either exogenous H_2S addition or genetic reduction of H_2S production have revealed that it possesses extensive anti-inflammatory[149], anti-atherosclerotic[150] and cytoprotective properties.[151] Recent work in a model of surgically induced critical hind limb ischemia also revealed the **pro-angiogenic** potential of endogenous H_2S upregulation (**Fig. 2**). In this study, short-term MetR promoted the post-injury growth of neo-vessels via increased expression of vascular endothelial growth factor (VEGF) and CGL activation, implicating endogenous H_2S upregulation as a novel therapy for PAD.[152]

Outline of this Thesis.

In (vascular) surgery practice during the pre-operative workup of patients there is are currently no distinct recommendations regarding their optimal nutritional state besides preventing malnutrition. Broadly, pre-, peri-, and post-operative guidelines for managing patient nutrition are described in the Enhanced Recovery After Surgery (ERAS) protocols[153], and implementation of ERAS in vascular surgery patients is linked with shorter in-hospital stays.[154] Concerning the pre-operative workup of patients scheduled for elective surgery, these guidelines recommend a "bodily state without malnutrition or malnourishment". The only other recommendation concerns ingestion of a carbohydrate drink ("carbohydrate loading") in the 24 hours before surgery to avoid a fasted state, and this is linked with improved glucose homeostasis.[155] In short, this points towards a potential gap in knowledge regarding optimal pre-operative dietary regimens, something this thesis aims to investigate. Is there room for a pre-operative dietary regimen that does not cause malnutrition, but can potentially benefit various peri- and post-operative vectors that together result in improved post-operative outcome?

Although the benefits of short-term DR have been established in several models of surgical stress and injury, whether these dietary regimens obstruct post-operative wound healing is undetermined. Therefore in **chapter 2** we tested two short-term isocaloric dietary interventions, namely protein restriction and methionine restriction, in a validated mouse model of surgical wound creation. We compared the time-to full wound closure in a McFarlane Flap wound model in both diets with an AL-fed cohort. Parallelly, we investigated the potential of these dietary interventions to improve glucose metabolism pre- and post-surgery.

Failure rates after vein graft surgery, in both coronary and lower extremity bypasses, remain high. Recently it was discovered that benefits of short-term DR seen in models of surgical IRI are derived from increased production of endogenous H_2S. But whether DR is also effective in vein graft surgery, and whether a reduction of total proteins (PR) can also upregulate endogenous H_2S is unknown. In **chapter 3**, we tested whether a short-term reduction in proteins can limit graft failure after rodent bypass surgery, and whether these benefits are also H_2S derived. We found that short-term PR attenuates graft disease, and that these benefits were CGL dependent. PR resulted in increased endothelial cell H_2S production, diminished VSMC migration and impaired neutrophil transmigration.

As a follow up of chapter 3 we study in **chapter 4** whether short-term MetR, which is feasible and doable in humans, can achieve similar benefits in rodent bypass surgery. Parallelly, there is uncertainty whether PVAT can play a protective role in VGD, or if it worsens outcomes. In the context of the interplay between adipose and DR, previous work indicated a shift towards an anti-inflammatory phenotype in inguinal adipose after MetR. However, the interaction between MetR and PVAT, and its influence on post-operative vascular remodeling remains undetermined. In **chapter 4** we not only test whether short-term MetR can protect from VGD and arterial intimal hyperplasia, but also investigate the link between MetR, PVAT and protection from VGD. Interestingly, we found that protection from VGD by MetR was PVAT-dependent, and that the interaction between MetR and PVAT also yielded a favorable cellular composition of the graft. In baseline/pre-op caval vein PVAT, short-term MetR upregulated AMPK-signaling and appeared to induce browning. Preconditioning with MetR also altered the PVAT response to surgical injury (bypass surgery), via a dampened pro-inflammatory response at post-op day 1.

Although short-term DR stands as a novel therapy to upregulate endogenous H_2S and improve remodeling after vascular injury, a preconditioning strategy via a diet may not always be feasible. Whether due to lack of diet-compliance or inadequate time available to precondition before undergoing surgery, so-called DR-mimetics are needed. In **chapter 5** we first tested the efficacy of exogenous H_2S in rodent arterial IH and VGD by supplementing drinking water with the

slow-releasing H_2S pro-drug GYY4137. Next, we dissolved GYY4137 in a Pluronic gel and enveloped arteries and vein grafts during the procedure. We elucidated the efficacy of this H_2S-gel in both arterial IH and VGD, while also interrogating its impact on the cells of the vascular wall. We developed a simple and safe H_2S-releasing gel that attenuates arterial and vein graft remodeling via a one-time, peri-procedural application. Mechanistically, we establish a link between this locally applicable DR-mimetic and VSMC migration, both *in-vivo* and *in-vitro*.

We and others have established the effectiveness of short-term DR in various preclinical models of surgical injury and stress. But whether this dietary intervention is feasible and safe in the vascular surgery patient population is unknown. In **chapter 6** we describe a first clinical trial performed in this direction. We enrolled several vascular patients scheduled for carotid endarterectomy on a 3-day PCR diet, while they resided in-hospital to closely monitor any adverse effects of the diet. Furthermore, we attempted to establish a clinical "discovery platform" regarding H_2S metabolism in humans and how the PCR diet impacts this. We report a possible link between H_2S producing gut-bacteria and short-term PCR.

This first attempt at short-term PCR in a clinical setting was performed in patients scheduled for elective carotid endarterectomy, who were required to stay in-hospital during the 3-day diet. As a follow-up towards that trial, for thesis we performed a second clinical trial. In **chapter 7** we describe the set-up and first experiences of a randomized clinical trial with short-term pre-operative PCR in elective vascular surgery patients. In this follow-up study in **chapter 7** we moved towards an out-patient setting and enrolled patients scheduled for any type of vascular surgery that involved an open wound. Furthermore, we extended the course of the diet to a 4-day diet and developed an extensive leukocyte subset flow panel to interrogate the innate and adaptive response to the PCR diet.

Both **chapter 6 and 7** highlight the very fortunate course of our work which enabled us to transition from rodent studies to vascular surgery patients, and essentially move our pre-clinical work from the lab bench towards the patient's bedside.

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Chapter 2.

Preoperative Protein or Methionine Restriction Preserves Wound Healing and Reduces Hyperglycemia.

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Abstract.

Background: Dietary restriction (DR), defined as reduced nutrient intake without malnutrition, is associated with longevity extension, improved glucose metabolism and increased stress resistance, but also poor wound healing. Short-term preoperative DR followed by a return to normal feeding after surgery results in improved surgical outcomes in preclinical models. However, the effect of preoperative DR on wound healing and perioperative glucose homeostasis is currently unknown. Here we tested the effects of two different pre-operative DR regimens, protein-restriction (PR) and methionine-restriction (MR), on wound healing and perioperative glucose homeostasis using an established murine model of wound healing in both non-diabetic and diabetic mice.

Materials & methods: Surgical outcomes were tested using the McFarlane flap in non-diabetic and streptozotocin-induced diabetic mice. Short-term dietary preconditioning included one-week of PR or MR diet (1-2 weeks) vs. an isocaloric complete diet prior to surgery; all mice were returned to a complete diet post-operatively. Outcome measures of flap wound recovery included skin viability and laser Doppler imaging of flap perfusion, and assessment of CD45+ cell infiltration. Glucose homeostasis was assessed by glucose tolerance testing and by perioperative glucose levels in the diabetic cohort.

Results: No significant differences were observed in percentage of viable skin, perfusion or immune cell infiltration at 7-10 days after surgery in PR or MR mice compared to controls in healthy or diabetic mice. Pre-operative glucose tolerance and post-operative glucose levels were however significantly improved by both PR and MR in diabetic mice.

Conclusion: Short-term dietary preconditioning with PR or MR did not impair wound healing in non-diabetic or diabetic mice. However, both regimens reduced pre-operative hyperglycemia in diabetic mice. Thus, brief preoperative dietary manipulations stand as strategies to potentially improve perioperative hyperglycemia with no deleterious effects on wound healing in mice.

1. Introduction.

Pre-clinical studies have demonstrated that brief alterations in dietary intake prior to surgery, collectively known as dietary preconditioning, improve outcomes in a variety of surgical injury models, including in models of renal ischemia or hepatic ischemia reperfusion injury, stroke and vascular intimal hyperplasia.[1-6] The underpinnings of these studies lie in the phenomenon of dietary restriction (DR), defined as reduced calorie intake without malnutrition, and first popularized by its ability to extend longevity in rodents. [7]

While DR has been at the forefront of ageing research, it also confers numerous other benefits in experimental organisms with perhaps even greater translational relevance. These benefits range from improved metabolic fitness to increased stress resistance. Importantly, DR has recently been shown to improve glucose and lipid homeostasis in humans. [8-11]

Classic DR regimens associated with longevity extension in rodents involve enforced food restriction of up to 60% of normal calorie intake (calorie restriction, CR). Alternate DR regimens that do not enforce food restriction include dietary protein restriction (PR) [12] or restriction of essential amino acids such as methionine (methionine restriction, MR) [13], suggesting that energy restriction per se is not necessary for DR-mediated longevity benefits. While molecular changes responsible for CR, PR and MR benefits and thus the degree of mechanistic overlap remain unclear, there is considerable phenotypic overlap amongst regimens, including the ability to precondition against a variety of surgical injury models. [2, 4, 5] Furthermore, PR and MR may be more clinically relevant over CR due to difficulties inherent to food restriction.

Despite pleiotropic benefits including maintenance of innate and adaptive immunity with aging [14], DR is also associated with negative outcomes including poor wound healing. [15-18] Importantly, dietary preconditioning regimens differ from DR regimens associated with poor wound healing or susceptibility to infection in their return to normal feeding immediately after surgery. Nonetheless, increased risk of wound healing problems or infection would significantly dampen translational potential in surgery, but have not been rigorously assessed in this context.

Wound healing is also compromised in diabetic patients, an increasingly clinically prevalent population, secondary to hyperglycemia and chronic inflammation.[19, 20] Consequently it is important to assess both the metabolic effects and wound healing potential of PR and MR in diabetic as well as non-diabetic models. Here, we tested the effects of PR or MR on wound healing and glucose homeostasis in a McFarlane flap wound healing model in the context of both normoglycemic and hyperglycemia in an streptozotocin (STZ) induced diabetic mouse model.

2. Materials & Methods.

2.1 Experimental Animals.

All animal experiments were carried out with the approval of the Harvard Medical Area Institutional Animal Care and Use Committee. 10-12 week old male C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME).

2.2 Diets.

For the non-diabetic cohort, after a 3-day acclimation period, mice were switched to a semi-purified nutritionally complete high-fat diet (HFD; 60% Kcal fat, 20% Kcal protein, 20% Kcal carbohydrate; Research Diets, New Brunswick, NJ) for two weeks. Cages were then randomized into the following isocaloric diet groups: control (complete HFD), PR (0% Kcal protein, 60% Kcal fat, 40% Kcal carbohydrates), MR (10% Kcal fat, 76% Kcal carbohydrate, 14% Kcal protein containing 0.05% methionine). Dietary preconditioning on PR lasted for 1 week, and MR for 2 weeks in the non-diabetic cohort and for 1 week in the diabetic cohort. A complete HFD was resumed immediately in all groups following surgery.

For the diabetic cohort, mice were injected with STZ (intraperitoneal, 50mg/kg) for 7 days, and after 10 days glucose measurements were obtained from venous tail blood. Mice were diagnosed as diabetic when fasting (4hr) glucose was >250mg/dl for 2 consecutive weeks. Mice were then randomized into same groups as above, and dietary preconditioning carried out for 1 week for all groups followed by complete HFD feeding immediately after surgery in all groups.

2.3 McFarlane Flap Surgery.

Briefly as previously described[21], mice were induced under 3% isoflurane and maintained under 1-2% isoflurane. The dorsal hairs were gently shaved and depiliated with Nair, and a 2.5 x 1.25cm area was measured on the dorsum just below the inter-scapular space. A scalpel was used to make the transverse incision and scissors for the remaining incisions to elevate a full-thickness peninsular flap. A sterile silicone sheet (0.14mm) was then placed inferior to the flap to prevent angiogenesis directly from below. The flap was approximated with interrupted 6-0 Vicryl sutures.

2.4 Laser Doppler Measures.

Laser Doppler (LDI, Moore Instruments, Axminster, United Kingdom) measures were performed pre-operatively, immediately post-operatively and then daily. Anesthetized mice were placed prone and maintained on continuous (1-1.5%) isoflurane anesthesia during scanning. Scan area was defined as only the area of the flap with approximately ~2mm of overlap with surrounding skin (flap scan area of X0:80, Y0:150, dX:35, dY:67) and a scan resolution of X:35 Y56 with scan speed of 4 ms/pixel. Data were analyzed using Moore LDI Review software V6.1.

2.5 Skin Viability Measures.

Daily digital photographs were taken while mice were under anesthesia for laser Doppler perfusion measurements. Flap viability/necrotic area was assessed and measured by a blinded surgeon with respect to gross appearance. The area of the flap was kept constant over all days assessed in the same mouse, the necrotic area was then traced and subtracted from the total surface area of the flap using Fiji software.

2.6 Glucose Measurements and Glucose Tolerance Test.

In the diabetic cohort, following the diagnosis of diabetes, daily non-fasting glucose was measured (AlphaTrak II) at the same time every morning during the week of dietary intervention. After 6 days of dietary precondition, mice were fasted for 4 hours and an oral glucose tolerance test performed (gavage, 1.5mg/kg glucose in 0.9% saline).

2.7 Harvest Tissue Processing and Histology.

Harvest of serum and tissues was performed at 7 or 10 days. Longitudinal sections of the flap were collected at the same position of the flap, fixed in 4% paraformaldehyde, embedded in paraffin, and stained with hematoxylin and eosin (H&E).

2.8 Immunohistochemistry and analysis.

Longitudinal flap sections of protein restricted and complete fed mice were stained with a CD45 antibody (rabbit anti-mouse, abcam10558) according to manufacturer's protocol. In short, slides were pre-incubated in a pressure chamber at 65ºC for 30 minutes and then deparaffinized. For antigen retrieval, slides were incubated in citrate buffer (abcam, ab94674) for 30 minutes at 95ºC and washed in PBS. Slides were pre-incubated with 10% goat serum (Vector, S-1000) for 45 minutes and stained overnight with the primary CD45 antibody at 4ºC. Next day slides were washed with PBS and stained with a biotinylated secondary antibody (goat anti-rabbit, Vector BA-1000) for two hours. After wash with PBS, slides were incubated in ABC-complex (Vector, PK6100) for 30 minutes, washed with PBS, stained with DAB peroxidase (Vector, SK4100) for 30 seconds, dehydrated and mounted with permount.

For Masson trichome histology staining 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

One cross section per mouse was imaged with a Zeiss Axio A1 microscope (Carl Zeiss) at 10x, and a flap overview was created by stitching photos with Photoshop (Adobe Photoshop 14.0). Resulting whole flap images were analyzed using the ImageJ (1.51p (Java 1.8.0_66) color deconvolution function. After thresholding each image, the percentage of CD45 positive area per whole flap was calculated or for collagen quantification the percentage of collagen area per whole flap was calculated and normalized to mm^2 of the flap.

2.9 Statistical Analysis.

All data are expressed as mean ± standard error of the mean. The Student T-test was performed for continuous variables and a p-value of less than 0.05 was considered significant. A 2-way ANOVA with multiple comparisons was employed when more than two groups were compared. All tests were performed with GraphPad Prism (7.0b).

3. Results.

3.1 Preservation of wound healing potential following short-term pre-operative protein or methionine restriction in non-diabetic mice.

Non-diabetic mice preconditioned on PR for 1week or MR for 2 weeks prior to surgery showed no impairment in percent viable skin compared to mice fed a complete diet (**Fig. 1**). Necrotic area was similar in both PR (n=8) and MR (n=8) mice compared to control mice fed a complete diet (n=8) (**Fig. 1A**). Laser Doppler perfusion imaging revealed a significant increase in mean flux intensity in PR mice on POD 1 (**Fig. 1A**, p=0.0189 vs. complete). The MR group also had a pronounced increase in skin perfusion pre-operatively, post-operatively and up to POD 5 (all p=<0.0001 vs. complete). However, no differences were observed at POD 7 in either PR or MR vs. complete diet controls (**Fig. 1A**).

Infiltration of leukocytes involved either in repair or in response to infection were also not significantly different between PR and complete diet fed controls (**Fig. 1G and I**). Also, there was no difference in collage content represented by collagen analysis after Masson trichrome staining of flaps (**Fig. 1H and J**). Thus, dietary preconditioning r preserved normal wound healing and immune infiltration in non-diabetic mice.

3.2 Preservation of wound healing potential following short-term pre-operative protein or methionine restriction in diabetic mice.

Similar results with regard to flap wound healing were observed in a cohort of STZ-induced diabetic mice (**Fig. 2**). No significant differences were observed in necrotic area between PR (n=11) or MR (n=11, one-week pre-op MR) and complete diet-fed diabetic mice (n=9). Interestingly, a significant increase in immediate post-operative flap perfusion (**Fig. 2B**, p=0.04) was observed in diabetic PR mice, consistent with the trend observed in non-diabetic PR mice. Importantly, there were no signs of infection that can accompany diabetic wound healing in any of the groups, nor any significant difference in leukocyte infiltration or collagen deposition between PR or complete fed mice (**Fig. 2F-I**).

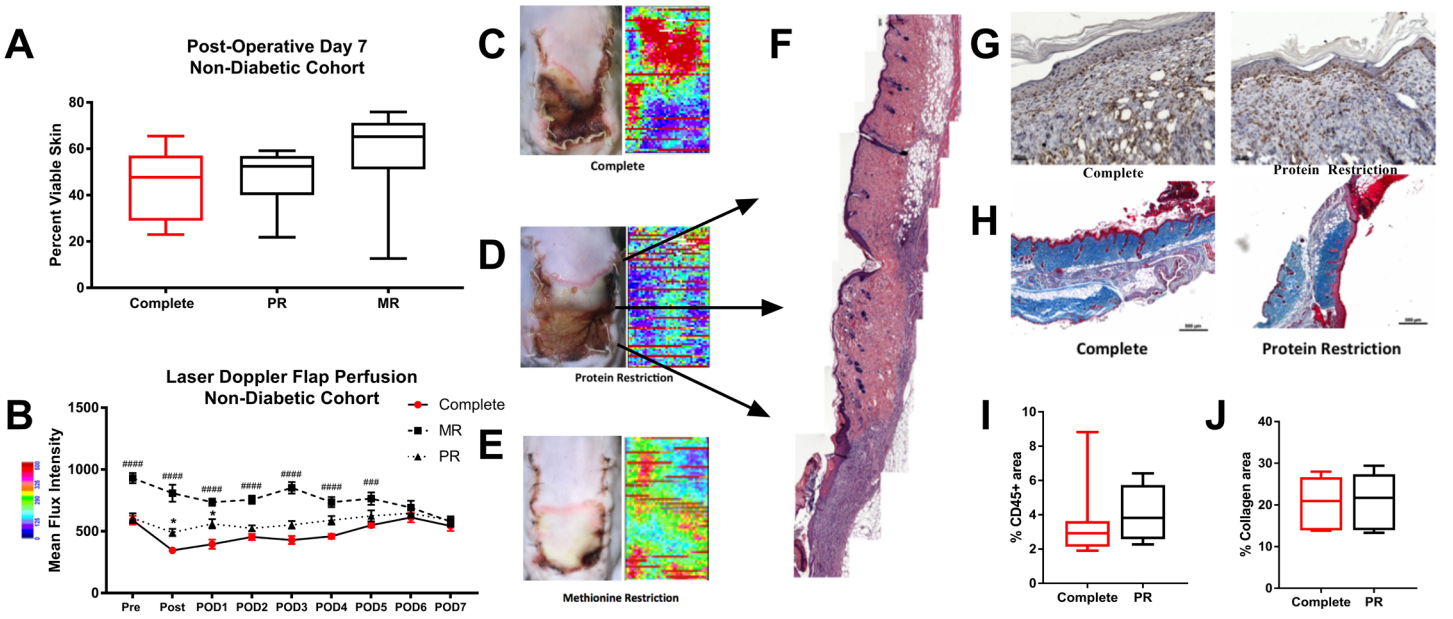


Fig. 1- Preservation of wound healing potential following short term pre-operative PR or MR.

(A) Percent viable skin in control (n=9), PR (n=8) and MR (n=10) mice revealing no significant differences on POD 7. (**B)** Laser Doppler flap perfusion measured pre (before surgery), post (immediately following surgery) and daily up to POD 7. Flap perfusion was increased in MR compared to complete group from pre-op up to POD 5 and in the PR group immediately post-surgery and on POD 1 compared to complete (2-way ANOVA) but was not significantly different amongst groups by day 6. **(C-E)** Representative images of flaps from complete, PR and MR mice on POD 7. Laser Doppler perfusion scans depict perfusion measurements. **(F)** Hematoxylin and eosin stained longitudinal flap section from a PR mouse with arrows depicting approximate location on flap section. **(G)** Representative images of skin flaps stained with CD45 (brown). **(H)** Representative images of skin flaps stained with Masson Trichrome for collagen. **(I)** Percent CD45 positive cells occupying area of flap (normalized to mm^2) in control (n=8) and PR mice (n=8). **(J)** Percent of area occupied by collagen (normalized to mm^2) in control (n=8) and PR mice (n=8). All data are represented as means ± standard error of the mean (* represents significant difference between complete and PR, # represents significant difference between complete and PR). (* P<0.05, *** P<0.001, **** P<0.0001)

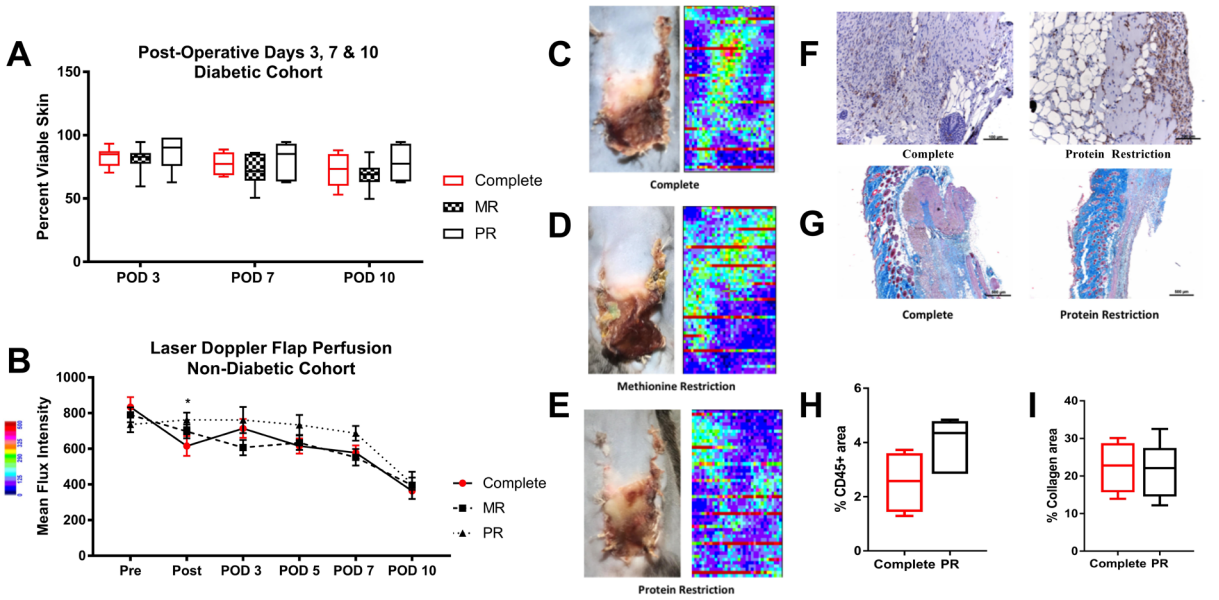


Fig. 2- Preservation of wound healing potential following short term pre-operative protein & methionine restriction in diabetic mice.

(A) Percent viable skin in control mice (n=9), PR (n=8) and MR (n=8) mice revealing no significant differences on days POD 3,7,10. (**B)** Laser Doppler imaging of flap perfusion measured pre (before surgery), post (immediately following surgery) and POD 3,5,7 and 10, showing increased perfusion in the PR group immediately post-surgery (2-way ANOVA). (**C-E**) Representative image of a control complete flap, a PR and MR flap at POD 7. **(F)** Representative images of skin flaps stained with CD45 (brown). **(G)** Representative images of skin flaps stained with Masson Trichrome for collagen. **(H)** Percent CD45 positive cells occupying area of flap (normalized to mm^2) in control (n=8) and PR mice (n=8). **(I)** Percent of area occupied by collagen (normalized to mm^2) in control (n=8) and PR mice (n=8). All data are represented as means ± SEM. (* represents significant difference between complete and PR) (*P<0.05)

3.3 Short-term preoperative protein or methionine restriction improves glucose tolerance and reduces perioperative hyperglycemia.

Although diabetes is a risk factor for impaired wound healing, it is also a concern for post-operative recovery. We thus monitored fed blood glucose levels and glucose tolerance as a function of dietary preconditioning during the perioperative period. In the diabetic cohort, PR rapidly and significantly reduced circulating glucose levels on days 1 and 3-7 of dietary preconditioning (**Fig. 3A**, p=0.009, p=0.006, p=0.049, p=0.006, p<0.0001, p=0.006) compared to control mice fed a complete diet. MR also significantly reduced circulating glucose levels on days 2-7 of dietary preconditioning (**Fig. 3A**, p=0.01, p=0.0004, p=0.001, p=0.001, p<0.0001, p=0.01 and p=0.048).

To further characterize glucose metabolism as a function of dietary preconditioning in diabetic mice, an oral glucose tolerance test was performed. PR improved glucose tolerance compared to control mice (**Fig. 3B**, 15 min. p=0.001, 30 min. p=0.005, 60 min. p=0.001, 120 min. p=0.002). MR also improved glucose tolerance at 60 and 120 minutes (**Fig. 3B**, p=0.01, p=0.002).

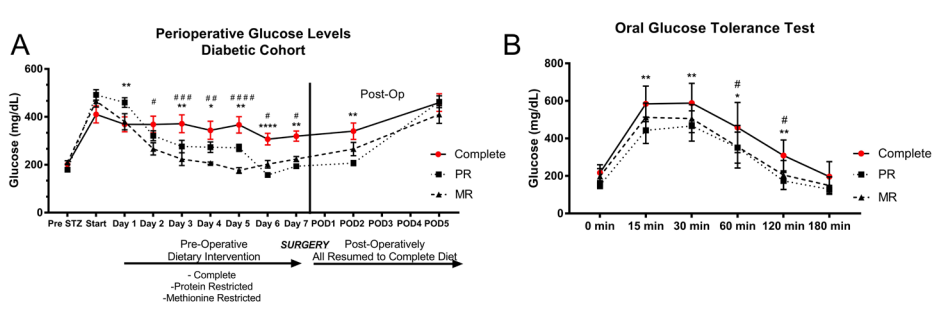


Fig. 3- Short term preoperative protein and methionine restriction improves perioperative circulating glucose and insulin levels.

(A) Non-fasted blood glucose in control, PR and MR mice during the perioperative period. Baseline (fasted, pre STZ) glucose measures are shown with rise in mean glucose levels in all groups (start) followed by glucose measures during the 7-day dietary intervention period as well as post-operatively up to POD 5. **(B)** Glucose tolerance test (1.5mg/kg) following one week of dietary intervention (2-way ANOVA). All data are represented as means ± SEM (* represents significant difference between complete and PR; # represents significant difference between complete and MR). (*/# P<0.05, **/## P<0.01, ### P<0.001, ****/#### P<0.0001)

Finally, because post-operative hyperglycemia is of concern, we monitored fed blood glucose level after resuming a complete diet. PR mice continued to have significantly lower blood glucose compared to controls at POD 2 (**Fig. 3A,** p=0.005).

4. Discussion.

There is currently a need for strategies to reduce risk of complications and improve outcomes in elective surgery. [22] Brief periods of DR prior to surgery followed by a return to normal food intake after surgery improve outcomes in a number of preclinical surgical models, thus representing a translational path forward to meet this demand. [4, 23, 24] Although anecdotal evidence based on multiple previous studies suggests that neither wound healing nor infection are increased in mice subject to dietary preconditioning, wound healing outcomes were never included as primary endpoints in the aforementioned studies. Here, we found no evidence of wound healing impairment in mice severely restricted in protein or the essential amino acid methionine before surgery however both diets significantly improved perioperative hyperglycemia. These data are also consistent with the results of clinical trials in humans subjected to various preoperative DR regimens that also show no apparent wound healing complications. [25, 26]

Previous preclinical studies of DR have shown impaired wound healing in part due to decreased collagen production *in vivo* and *in vitro*. [15-18] The discrepancies between this study and previous efforts to characterize the effects of DR on wound healing are likely due to the differences in dietary paradigms. In the dietary preconditioning paradigm, animals are refed a complete diet after surgery, while in previous studies DR was maintained both before and after surgery. [15-18] However, differences could also be due the models, as our pedicle flap model is a severe wound healing model relying predominately on angiogenesis for flap survival rather than collagen formation and wound contracture. Interestingly, the increase in perfusion measured by laser Doppler specifically in the MR group is consistent with our recent finding that MR increases angiogenesis.[27] Furthermore, the difference between MR and PR in the non-diabetic mice, as well as within MR groups between non-diabetic and diabetic cohorts, could be related to the duration of MR, which was 2 weeks only in the non-diabetic group in which increased perfusion was observed. Nonetheless, while this did not increase wound healing in this group, we conclude that neither MR nor PR worsen wound healing in the McFarlane flap model.

Various surgical subspecialties have shown a relationship with perioperative hyperglycemia in diabetic patients and wound complications.[28, 29] Although the relationship between perioperative hyperglycemia and wound complications is not yet clearly defined many studies have found positive correlations [30, 31], including a recent retrospective study pointing to a significant association between perioperative glucose measures above 200 mg/dl and increased rates of dehiscence.[29] Furthermore, current Enhanced Recovery After Surgery guidelines recommend consumption of carbohydrate-laden beverages in the perioperative period specifically to reduce post-operative hyperglycemia.[32] Here, we observed improved glucose tolerance and a reduction in perioperative glucose, including in the PR group on day 2 after a return to a complete diet, in diabetic mice made hyperglycemic by STZ pretreatment. Thus, in addition to the lack of negative effects on wound healing, dietary preconditioning improved glucose homeostasis and post-operative hyperglycemia in a diabetic mouse model.

5. Conclusion.

Here we show that dietary preconditioning regimens previously associated with improved surgical outcomes do not impair wound healing in non-diabetic or diabetic mice. Moreover, we found that these diets improved perioperative glucose tolerance and perioperative hyperglycemia in diabetic mice. In conclusion, brief dietary manipulations stand as simple strategies to potentially improve perioperative hyperglycemia with no deleterious effects on wound healing in mice, thereby further enhancing clinical applicability.

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5.2 Disclosures.

The authors declare no conflicts of interest.

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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

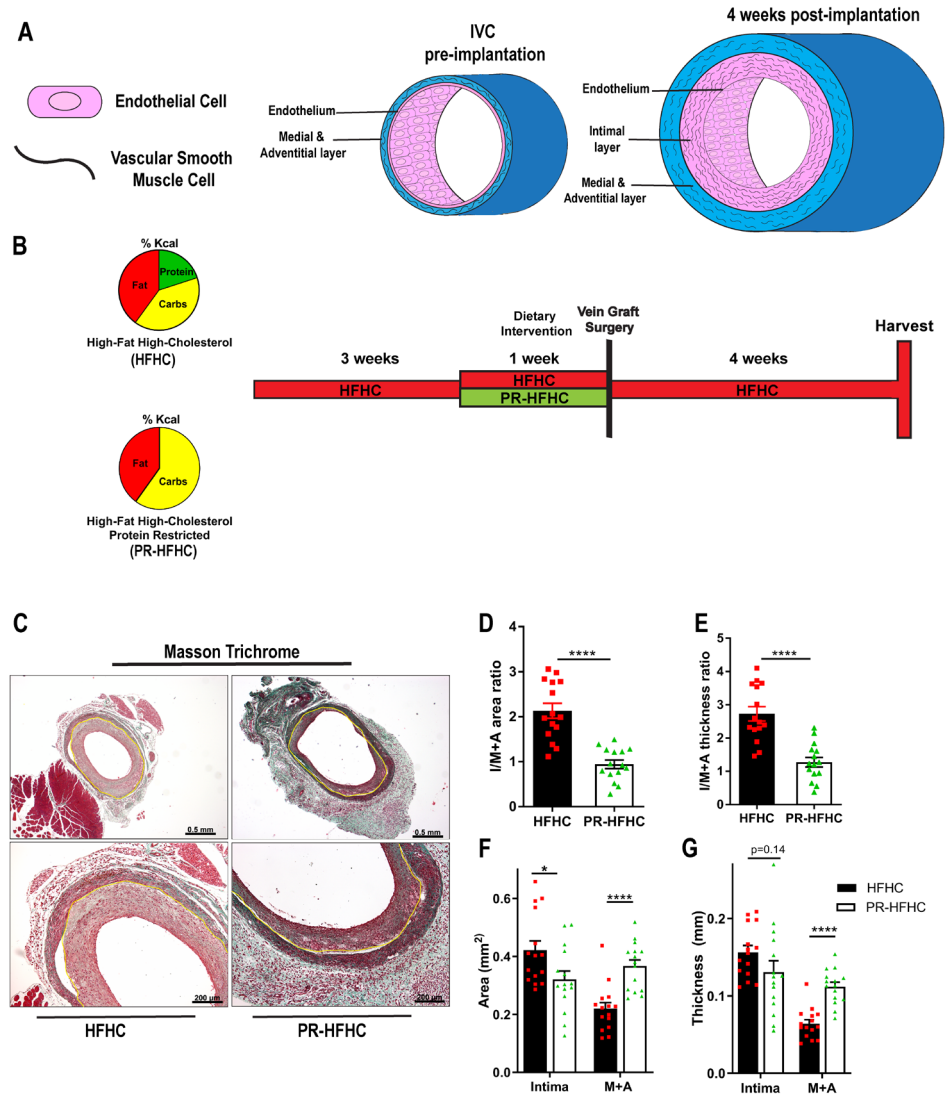


Figure 1. Short-Term Protein Restriction Attenuates Vein Graft Disease.
A: Schematic representation of the development of intimal hyperplasia 4 weeks after implantation of a vein graft (inferior vein cava, IVC) from a separate donor mouse. **B:** Schematic representation of diets (left) and dietary alterations (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; *$p<0.05$, ****$p<0.0001$.

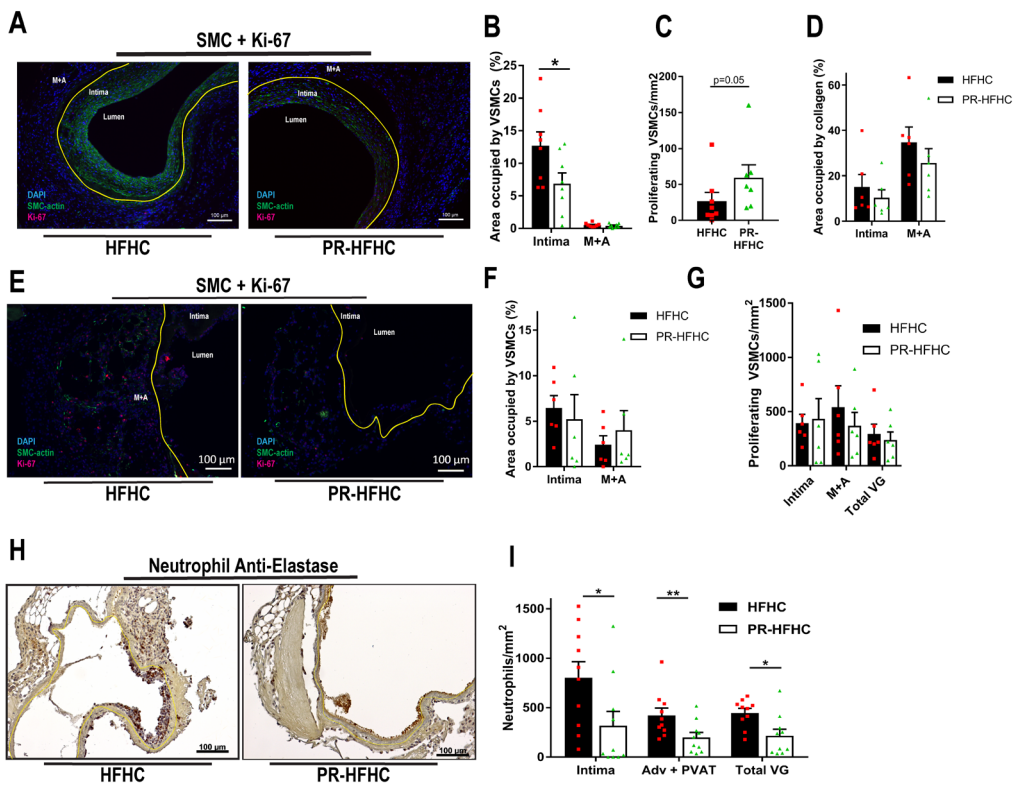


Figure 2. Preoperative Protein Restriction Limits Smooth Muscle Cell Migration and Inhibits Leukocyte Transmigration.

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

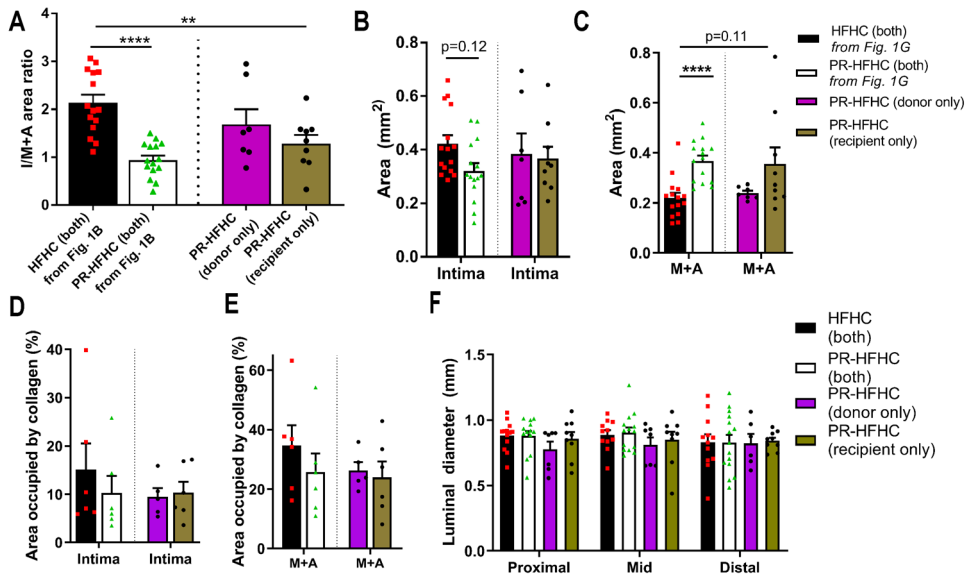


Figure 3. Contribution of Donor and Recipient Response to Protein Restriction in Attenuation of Vein Graft Disease.

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.

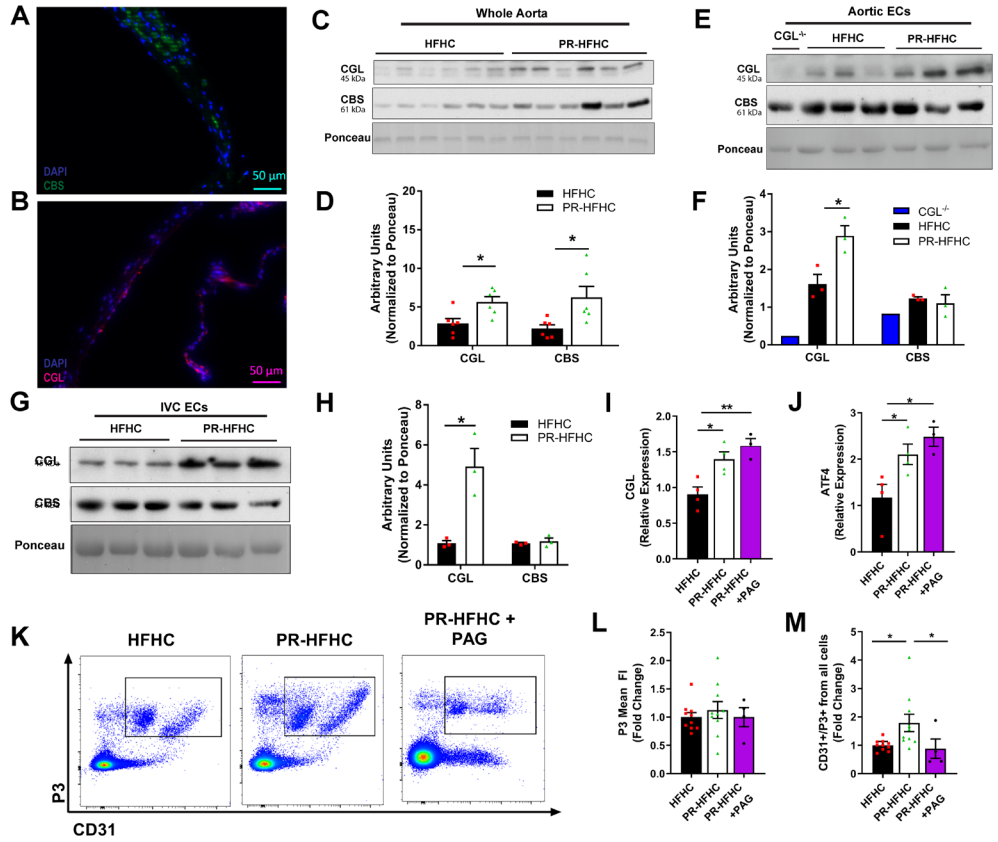


Figure 4. Protein Restriction Upregulates Cystathionine Gamma-Lyase and H_2S Levels in Endothelial Cells.

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

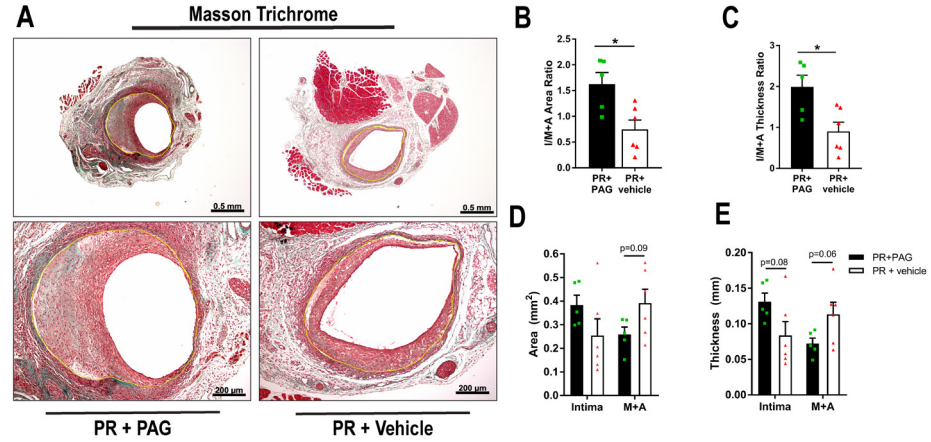


Figure 5. Cystathionine Gamma-Lyase is Required for Protein Restriction Mediated Attenuation of Intimal Hyperplasia.

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**).

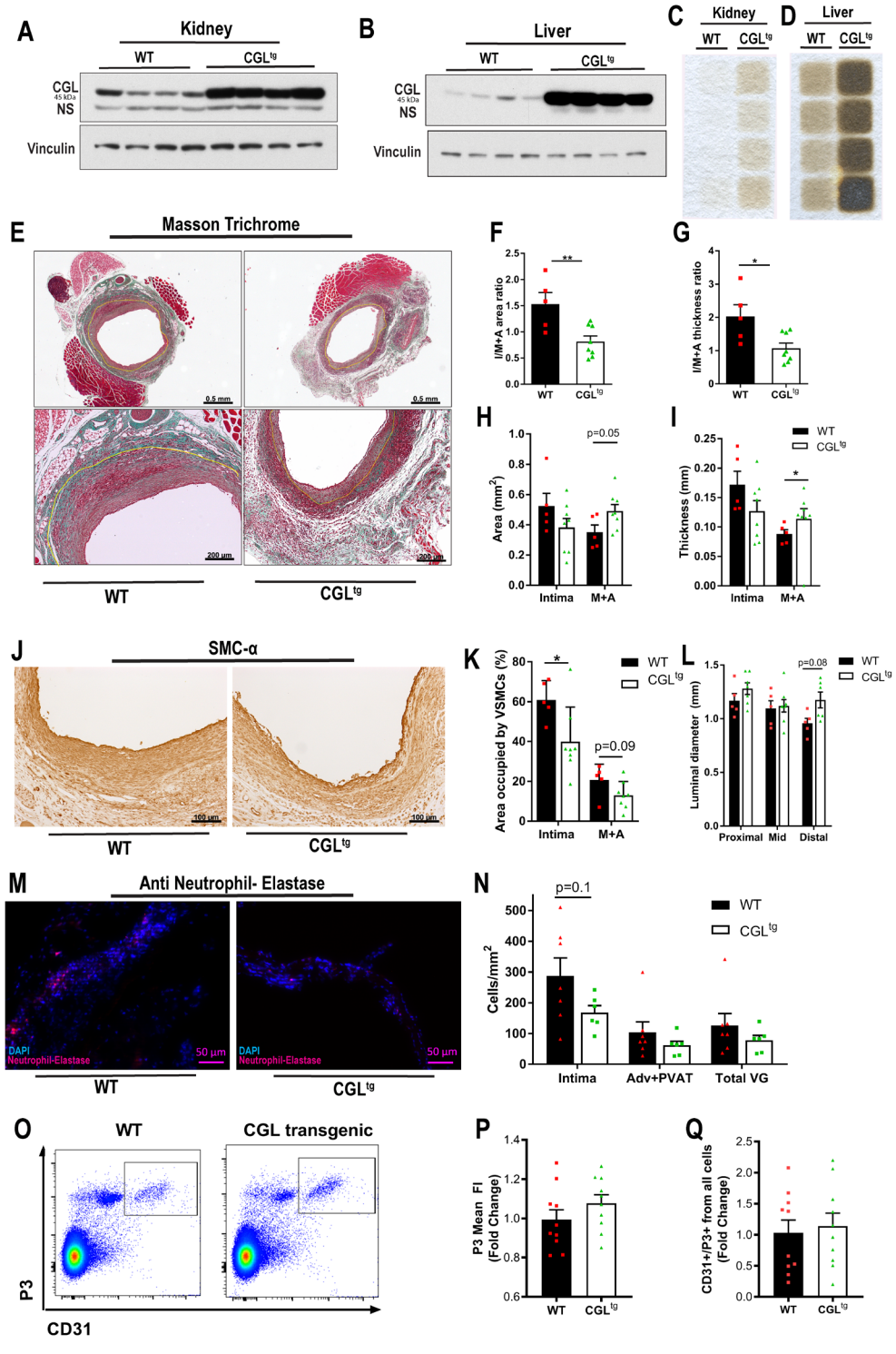


Figure 6. CGL Overexpression Protects Against Vein Graft Disease But Does Not Increase Basal Endothelial Cell H_2S Levels.

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.

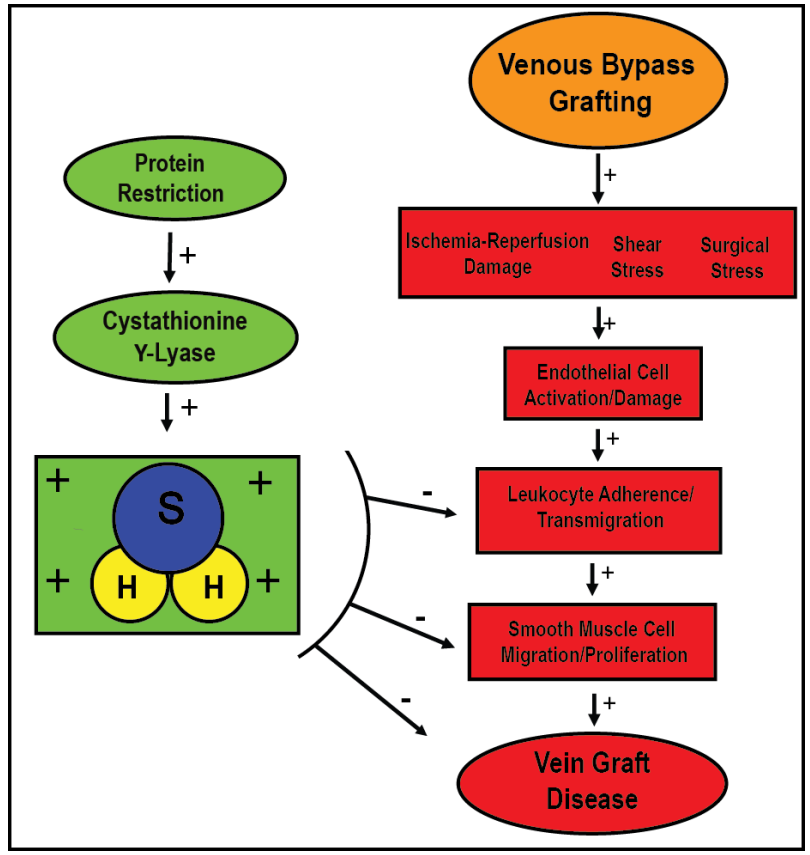


Figure 7. Model for attenuation of vein graft disease by short-term protein restriction via upregulation of cysthathionine-Y-lyase.

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.

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Chapter 4.

Short-Term Pre-Operative Methionine Restriction Protects from Vascular Wall Maladaptation Via PVAT Dependent Mechanisms.

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Abstract.

Rationale: All (cardio)vascular procedures involve peri-operative manipulation of adipose tissue, either subcutaneous or perivascular (PVAT), while a "sick fat" phenotype is detrimental to surgical outcome. Short-term preoperative methionine restriction (MetR) shows promise as a novel strategy to alter the response to surgical injury, but its effects in arterial and vein graft intimal hyperplasia (IH) are unknown.

Objective: We hypothesized that short-term MetR can attenuate IH after arterial injury and vein graft surgery, and that these benefits are PVAT-dependent.

Methods and Results: Mice (C57BL/6 male, 12-weeks) consumed a high-fat diet (Control; 0.6% methionine/60% fat) for 3-weeks. Half were switched to a 1-week MetR diet (0.05% methionine/60% fat) while the control group remained on control-diet. One cohort from both diet groups underwent carotid artery focal stenosis. Donor-animal vena cava (VC) PVAT was either partially stripped, completely stripped (-PVAT) or left intact (+PVAT), then carotid-interposition grafting into diet-matched recipients was performed. Immediately post-op, all mice were control-fed. At post-op day (POD) 28, grafts and carotid arteries were harvested for histology and immunohistochemistry. Pre-op (VC, aorta [AO]) and vein graft POD1 PVAT were processed for RNA-sequencing.

Results: Short-term MetR attenuated intima/media-area ratios after focal stenosis. In vein grafts, protection was dependent on diet-PVAT interactions, with a 53.3% decrease in intima/media+adventitia-area ratios in the MetR +PVAT group, compared Control +PVAT. MetR +PVAT vein grafts had a 59.1% reduction in intimal M1/M2 ratios, compared to Control +PVAT, and this was also dependent on diet-PVAT interaction. MetR increased thermogenesis in arterial-, and AMPK-signaling in arterial and venous PVAT. At POD1 MetR downregulated the pro-inflammatory ligand tenascin-c and anti-atherosclerotic enzyme lysyl-oxidase.

Conclusions: Short-term preoperative MetR attenuated arterial and vein graft IH, and in vein grafts this was PVAT dependent. Mechanistically, MetR induced browning and increased AMPK-signaling in PVAT at baseline, while dampening the post-operative pro-inflammatory response to the surgery.

1. Introduction.

With more than 1 million inpatient (lower extremity) vascular procedures[1] and 150,000 coronary artery bypasses performed annually in the US alone[2], revascularization surgery remains a mainstay in the treatment of arterial occlusive disease. These interventions, however, are hampered by high failure rates. One-year primary patency after lower extremity balloon angioplasty, for example, ranges between 31-55%.[3, 4] And while venous bypasses of the coronary artery have a one-year primary patency of 76%[5], in the lower extremity this falls towards 60%.[6]

The mid- to long-term failure of an initial successful vascular procedure originates from accelerated intimal hyperplasia (IH).[7] This pathophysiological response to surgical injury as in balloon angioplasty, or altered hemodynamics as in vein grafting, is defined by initial endothelial dysfunction and leukocyte transmigration.[7, 8] This in turn triggers vascular smooth muscle cell (VSMC) migration and proliferation and ultimately occludes the artery or vein graft.[7, 9] And despite decades of research, therapies to limit this remodeling response of the vascular wall after a (cardio)vascular intervention are not available.

In addition to the interaction between systemic inflammation and the cellular composition of the vascular wall, perivascular adipose tissue (PVAT) surrounding the vessel also functions as a paracrine organ with the potential for impacting vascular vessel patency following an intervention.[10, 11] Interestingly, in the context of obesity and subsequent inflamed PVAT, preclinical work suggests that this can accelerate IH.[11] For example, high-fat feeding (HFD) in rodents increases the secretion of interleukin-6, interleukin-8 and monocyte chemoattractant protein-1 (MCP-1) from perivascular adipocytes.[12] A comparable diet triggered the infiltration of pro-inflammatory monocytes in visceral adipose tissue which was then transplanted onto the carotid artery, as a surrogate for the presence of inflamed PVAT pre-surgery. After carotid wire injury, cohorts who received inflamed adipose tissue had exacerbated neointima formation.[13] In a comparable study, aorta PVAT transplantation onto the carotid artery from HFD-induced obese mice also accelerated neointima formation subsequent to carotid wire injury.[14] Switching from a HFD to normal chow can also rapidly alter fat phenotype[15], but the functional relevance of this in terms of protection against IH as well as a underlying mechanism of action remain uncharacterized.

Surgical trauma to the fat itself can also alter its local phenotype and even yield a systemic response. For example, mechanical injury to subcutaneous adipose tissue triggered local production of interleukin-6 and interleukin-1β (IL-1β).[16] In a separate study, a comparable surgical injury to the fat elicited increased levels of circulating interleukin-6, and this response was exacerbated in obese patients.[17] In a preclinical model of surgical injury to the adipose tissue, unilateral surgical trauma to inguinal adipose induces local and distant browning[15], but whether this could be beneficial in post-operative recovery is unknown.

In this same surgical trauma model, the authors lowered the high-fat content of the diet to normal levels for the 3-weeks leading up to the surgical injury. Interestingly, this short-term change in dietary intake not only reduced baseline adipose levels of MC-1 and tissue necrosis factor-α (TNF-α), but it also altered the response of the fat to surgical trauma. At post-op day (POD) 1, local levels of both IL-1β and TNF-α where reduced compared to mice who received a preoperative high-fat diet.[18] Which pathways are activated due to such a short-term change in dietary intake, and whether this protection could extend to PVAT in vascular injury models, has not been investigated.

This concept of utilizing short-term dietary interventions before surgery to precondition the body, in order to alter the host's response to surgical injury, stands as an emerging approach to enhance surgical outcomes.[19, 20] Dietary restriction (DR), defined as restriction of either total calories, macronutrients such as protein or specific essential amino acids without malnutrition, during the preoperative time period of days to weeks represents one such dietary preconditioning approach.[20, 21] Efficacy of these short-term diets has been shown in a wide range of preclinical surgical models, including renal[19, 22, 23], hepatic[19, 24, 25] and vascular injury models.[26] Recently, we reported that short-term restriction of dietary protein intake attenuates IH and subsequent vein graft disease (VGD) in a venous bypass graft model, potentially via impaired VSMC migration.[27]

Methionine restriction (MetR) is a DR regimen in which dietary sulfur amino acid (methionine and cysteine) content, but not overall calorie intake, is reduced. In rodents, MetR has pleiotropic beneficial effects on markers of cardiometabolic health[28] and lifespan[29] likely via effects on adipose tissue[30] and energy metabolism[31], while specifically in surgical models MetR improves outcome of femoral ligation by increasing angiogenic potential[32] and preserves wound healing[33]. In humans, MetR delivered for up to 16 weeks as a semi-synthetic diet is feasible and increases fat oxidation and reduces intrahepatic lipid content.[34]

Here we tested the hypothesis that short-term pre-operative MetR can attenuate IH in models of either arterial injury or VGD specifically through interaction with PVAT, with implications for dietary preconditioning in human vascular injury. Mechanistically, we evaluated changes in PVAT gene expression prior to and after surgery and their modulation by MetR.

2. 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 () and in accordance with the NIH guidelines. All surgical experiments were performed on C57BL/6 mice (male, 14-16 weeks old, Stock No: 000664, Jackson Laboratory). 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 Dietary Intervention.

All mice (aged 10-12 weeks old) were started on a 3-week 60% fat (by calories), 0% cysteine diet (Research Diets, A18013001) [Control] which contained standard levels of methionine (0.64%, 2.6% of total protein)., (**Fig. S1A**) After 3 weeks of Control diet, 1 cohort was switched to a methionine restriction (MetR) diet containing 60% fat, 0% cysteine and 0.07% methionine0.3% of total protein) [Research Diets, A18022602]. (**Fig. S1A**) After 1 week of MetR or continued Control diet, mice were either harvested for caval vein /baseline studies or underwent a surgical intervention (see below for description of vein graft surgery and focal stenosis creation. Immediately post-operatively, all mice were switched back to the Control diet and harvested at either post-op day 1 (POD1) or post-op day 28 (POD28). Mice subjected to MetR lost approximately 20% of their starting weight (**Fig. 1SB,** representative weight curve), despite hyperphagia during the dietary intervention (**Fig. S1C**), but regained weight rapidly post-operative (**Fig. 1SB**).

2.3 Focal Stenosis Creation.

A focal stenosis was created as described previously to generate an arterial intimal hyperplastic response.[35] Mice were anesthetized with isoflurane and the right common carotid artery (RCCA) was dissected from its surrounding tissue. A 35-gauge blunt needle mandrel was then placed longitudinally along the RCCA and tied with a 9-0 nylon suture approximately 2-2.5mm proximal to the bifurcation. After removal of the needle mandrel, the skin was 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 Vein Graft Surgery.

Vein graft surgery was performed as described previously. [36] In brief, anesthesia was induced via a nose cone with 5% isoflurane and maintained under 2-3% for the duration of the procedure. Shortly before the start of the recipient surgical procedure, the thoracic caval vein of a donor mouse was harvested and placed in ice-cold sterile 0.9% NaCl supplemented with heparin (100UI/mL). In the recipient, neck region fur was removed, and a neckline incision was performed. The right common carotid artery (RCCA) was dissected from its surrounding soft tissues and 2 8-0 nylon sutures were tied in the middle, approximately 1mm apart. RCCA was then cut between the two sutures to facilitate an end-to-end anastomosis. The proximal and distal RCCA was then everted over an autoclavable nylon cuff (Portex) of approximately 2mm while clamped with vascular clamps. The everted carotid walls were secured with an 8-0 nylon suture. Next the donor caval vein was sleeved between both RCCA ends, and an end-to-end anastomosis was created with 8-0 nylon sutures. The distal followed by the proximal vascular clamp was released to restore blood flow. 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).

2.5 Vein Graft PVAT Manipulation.

In routine rodent vein graft surgery[36], as well as in our first cohort of vein graft dietary intervention experiments, the donor caval vein was partially trimmed of its surrounding PVAT to facilitate ~~technically straightforward~~ end-to-end anastomosis creation in the recipient. In a follow-up cohort of C57BL/6, we either completely stripped the donor caval vein of its surrounding PVAT or left all PVAT intact. The donor caval vein (with/without PVAT) was then transplanted into a recipient RCCA on a corresponding diet (Control-fed or MetR), to create a vein graft with PVAT intact, or a vein graft lacking PVAT. This resulted in 4 separate groups evaluating the interplay of diet and the presence of PVAT around the vein graft: Control – PVAT, Control + PVAT, MetR – Control, MetR + PVAT. Stripped donor caval vein PVAT was collected on dry ice, snap frozen in liquid nitrogen and then stored at -80°C for subsequent analyses.

2.6 Vein graft/RCCA POD28 Harvest.

Mice were exsanguinated under anesthesia, followed by insertion of a 21G needle in the left ventricle. Whole-body perfusion was performed with lactate Ringers solution for 3 minutes, then switched to 3 minutes of perfusion-fixation with 10% formalin. The graft/RCCA was excised en-bloc via a midline neck incision and transferred to a 10% formalin (in PBS) solution for 24 hours. After 24 hours the tissue was transferred to a 70% ethanol solution for further processing.

2.7 Baseline Studies.

To study effects of diet without surgery, baseline data was secured from mice fed an identical dietary intervention (3-week control diet followed by 1-week MetR or control) and harvested after 1 week of MetR/control. All tissue was collected on dry ice and snap frozen in liquid nitrogen, before storage at -80°C. For baseline harvest, mice were first anesthetized, and a cardiac puncture was performed to collect 1mL of whole blood in a 1.5mL ethylenediaminetetraacetic acid (EDTA)-coated Eppendorf for downstream blood analysis. A thoracotomy was performed and caval vein PVAT was carefully dissected from the vessel wall with forceps. The caval vein was then harvested and caval vein wall and PVAT were stored separately. Lungs and heart were removed and thoracic aorta PVAT was carefully separated from the vessel wall, followed by harvest of the aortic wall. Both aorta and aorta PVAT were stored separately.

2.8 Vein Graft POD1 Harvest.

For vein graft POD1 harvest, after mice were anesthetized, the neck suture was removed and the surgical field from the previous day was opened. Vein graft patency was first ensured. In one cohort of mice, 1mL whole blood was collected via cardiac puncture in an EDTA-coated Eppendorf for downstream whole-blood analysis, and then the vein graft was removed for histology as follows. A thoracotomy was performed, followed by whole-body perfusion via the left ventricle with ringer's lactate solution. A 21G syringe containing O.C.T. (Tissue-Tek, # 25608-930) was carefully inserted in the brachiocephalic trunk oriented towards the RCCA. Next, O.C.T. was slowly released into the

brachiocephalic trunk until the vein graft started to dilate. After placing an 8/0 suture around the distal cuff, it was tightened followed by a second suture around the proximal cuff. The now dilated vein graft was removed en-bloc and placed in a mold filled with O.C.T., then placed on dry ice and stored in -80°C. In a second cohort of mice, the PVAT surrounding the vein graft was carefully removed using forceps and collected in a 1.5mL Eppendorf on dry ice. Next, the vein graft wall itself was taken out and collected in a separate Eppendorf on dry ice. Both PVAT and vein graft were then snap frozen in liquid nitrogen and stored at -80°C.

2.9 Vein Graft/RCCA Histology.

POD28 Vein grafts and RCCA (at POD28 after focal stenosis surgery) were harvested, embedded in paraffin and cut in 5µm sections by microtome, then mounted on slides. Grafts were sectioned at regular intervals of 200 µm, starting from the proximal cuff till 1000µm post proximal cuff. Focal stenosis arteries were cut at regular intervals of 400µm, starting at 400µm proximal from the focal stenosis, until 2800µm proximal from the stenosis. For histomorphometric analysis, a Masson-Trichome staining was performed: after deparaffinization to 95% ethanol, slides were immersed in 5% picric acid (in 95% ethanol) for 3 minutes, followed by a 3-minute stain in working Harris Hematoxylin Solution (Fisher 213 Scientific, cat# 245-678). After a brief tap water wash, slides were stained with 1% Biebrich Scarlet in 1% acetic acid (Fisher Scientific, cat# A38S-500) for 3 minutes, followed by a quick rinse in distilled water. Slides were then stained for 1 minute in 5% Phosphomolybdic/Phosphotungstic acid solution and immediately transferred to 2.5% light green SF yellowish in 2.5% acetic acid (Fisher Scientific, cat# A38S-500) for 4 minutes. Followed by a quick rinse in distilled water and a 2-minute rinse in1% acetic acid solution (Fisher Scientific, cat# A38S-500). After dehydration with Xylene slides were covered with a cover glass employing Permount (Electron Microscopy Science, cat# 17986-05). Brightfield images of vein graft and carotid artery cross-sections were taken with a Zeiss Axio A1 microscope (Carl Zeiss). Histomorphometric analysis was performed using Image J 1.51p (Java 1.8.0_66) (see below).

2.10 Histomorphometric Analysis.

For vein graft histomorphometric analysis, images of cross sections taken at 200µm, 400µm, 600µm, 800µm and 1200µm post-cuff were uploaded in ImageJ. Per distance, 1 cross section was analyzed. Area and perimeter of lumen, internal elastic lamina and adventitial border were measured in $\mu m^2/\mu m$. Next, lumen area, intimal area, intimal thickness, media+adventitia (M+A) area, M+A thickness, intimal/media+adventitia (I/M+A) area ratio and I/M+A thickness were calculated as described previously.[37] 200µm-1000µm cross sections were next averaged into a per-vein graft histomorphometric endpoint. For focal stenosis histomorphometric analysis, RCCA cross section images taken at 400µm, 800µm, 1200µm, 1600µm and 2000µm proximal from the focal stenosis were uploaded in ImageJ. Lumen area and perimeter, internal elastic lamina area and perimeter, external elastic lamina area and perimeter were measured. Next, lumen area, intimal area and thickness, medial area and thickness and I/M area and thickness ratios were calculated. Collagen measurements were performed via the color deconvolution function in ImageJ, the resulting split green area was measured via pixel-threshold and normalized to total intimal/M+A and total vein graft area in %.

2.11 Immunohistochemistry.

First, vein graft slides were incubated for 30' at 60°C in a vacuum oven, followed by immediate deparaffinization. Next, antigen retrieval was performed for 30' at 97°C in Citrate buffer (pH 6.0, in PBS) [Abcam, ab93678]. Then, slides were pre-incubated with 10% goat serum (Life Technologies, 50062Z) in PBS with 0.3M Glycine (Aijomoto, R015N0080039) for 1hr at room temperature (RT). Slides were then incubated with primary antibodies. For VSMC+KI-67 double-staining: SMC-α (mouse anti-mouse, Abcam, ab7817, 1:800) and Ki-67 (rabbit anti-mouse, Abcam, ab16667, 1:100) o/n at 4°C. For M1/M2 macrophage staining, 1 vein graft slide containing 2-4 cross sections was double stained with the general macrophage marker Mac-3 (rat anti-mouse, Fisher Scientific, B550292, 1:600) and either iNOS (rabbit anti-mouse, abcam, ab3523, 1:100) for M1, or CD206 (rabbit anti-mouse, abcam, ab64693, 1:800) for M2. Slides with primary antibodies were incubated o/n at 4°C. Slides were then washed in PBS + tween (PBST) and incubated in secondary antibody for 2hrs at RT. For SMC-α + Ki-67 double staining, slides were incubated with Alexa Fluor 647 (goat anti-mouse, A-32728) and Alexa Flour 568 (goat anti-rabbit, A-11011) at 1:600. For M1/M2 staining, slides were incubated with Alexa Fluor 568 (goat anti-rabbit, A-11011) and Alexa Fluor 647 (goat anti-rat, A-21247) at 1:600. After secondary antibody incubation, slides were washed in PBST and mounted with DAPI (Vector, CB-1000) or stained with Hoechst staining solution and mounted with anti-fade as indicated.

2.12 Immunohistochemical analysis.

For fluorescent IHC, 20x images were taken with a Laser Scanning Confocal Microscope (Zeiss LSM800) and automatically stitched. For non-fluorescent IHC, 10x brightfield images were taken with a Zeiss Axio A1 microscope (Carl Zeiss)

and stitched with Adobe Photoshop. Lumen area, intimal area, M+A area and PVAT area were defined and measured in ImageJ, based on the corresponding Masson-trichome histology picture. For VSMC + Ki-67 double staining analysis, 1 cross section per vein graft was analyzed (600 or 800µm). Based on Hoechst/DAPI stain the lumen, intima and M+A area was measured in mm^2. The SMC-α positive fluorescent (647nm) channel was analyzed for total SMC-α positive pixels via color-threshold and then normalized to its respective vein graft layer (intima, M+A or total vein graft) in %. A cell positive for both SMC-α and Ki-67 was regarded as a "proliferating VSMC". Total proliferating VSMCs per vein graft layer were then counted and normalized to cells/mm^2. Total SMC-α positive cells were counted per vein graft layer and normalized to VSMC/mm^2 per vein graft layer. For analysis of macrophage polarization, one slide per vein graft (at 800µm) containing 2-4 cross sections was stained with Mac-3+iNOS or Mac-3+CD206. Lumen area, intimal area, M+A area and PVAT area were measured in mm^2. All cells positive for Mac-3 were determined macrophages (M_θ). Cells positive for Mac-3 and iNOS: M_1-macrophage. Cells positive for Mac-3 and CD206: M_2-macrophage. In each cross section the total number of M_θ and M_1 or M_2-macrophages per vein graft layer was counted and normalized to mm^2. Per vein graft layer, M_1 and M_2-macrophages (in count per mm^2) were normalized as a percentage of the total M_θ-macrophages present in that layer (also in count/mm^2). The resulting M_1 and M_2-macrophage percentages were then used to calculate a M_1/M_2 ratio.

2.13 Duplex ultrasound biomicroscopy

In both MetR and control-fed mice subjected to vein graft surgery with/without PVAT, high resolution ultrasonography was performed at POD14 and POD28. A Vevo 2100 imaging system with 18- to 70-MHz linear array transducers (Visual Sonics Inc., Toronto, ON, Canada) was employed to measure vein graft lumen diameters. Mice were anaesthetized with 2-3% isoflurane and body temperatures were maintained at 37°C using a heated stage. M-mode was used for vessel cross-sectional dimensions. Three luminal axial images were performed (proximal, distal, and mid vein graft), and mean vessel luminal diameters were calculated.

2.14 RNA isolation from adipose tissue & primary cells.

Adipose tissue samples were collected on dry ice, snap frozen in liquid nitrogen and stored at -80°C. For RNA sequencing analysis of aorta (arterial) PVAT, the aorta PVAT of two mice on corresponding diets was pooled in one pre-cooled Eppendorf on dry ice. For caval vein and vein graft PVAT RNA sequencing, PVAT from 3 mice on corresponding diets was pooled in one pre-cooled Eppendorf on dry ice. Next, 250 µL Trizol (Thermo Fisher, cat# 15596026) was added per Eppendorf and the tissue was thoroughly homogenized with a hand-held tissue homogenizer. After homogenization, samples were centrifuged at 12.000x*g*, 5 min at 4°C. Supernatant was transfer to a fresh tube and incubated for 5' at RT, then 200µL of chloroform (Sigma-Aldrich, cat#288306-1L) was added and the tube incubated for 2' on wet ice. After centrifuging tubes at 12.000x*g* for 10' at 4°C, the aqueous layer was collected on a fresh tube on ice. 250µL iso-propanol and 1µL glycogen was added to each sample, vortexed and centrifuged (12.000x*g*, 10' at 4°C). Supernatant was aspirated and 75% EtOH was added before Eppendorf was vortexed and centrifuged (12.000x*g*, 10' at 4°C). This EtOH wash was repeated for a total of three times, then the RNA-pellet was left to dry for 20' at RT. Pellet was then eluted in 20µL of RNAase free H_2O and stored at -80°C.

2.15 Statistical analysis.

All data are expressed as mean ± standard deviation unless indicated otherwise. Normality testing was performed employing the Shapiro-Wilk normality test. Normally distributed data was analyzed by Student's *t*-test or two-way ANOVA. Non-normally distributed data was analyzed by Mann-Whitney test. All testing was done via Graphpad Prism (8.4.2).

3. Results.

3.1 Short-term MetR improves both arterial and vein graft revascularization strategies.

We first examined whether MetR was able to attenuate negative wall remodeling after an intimal hyperplasia-inducing hemodynamic vascular procedure by creating a focal stenosis of the RCCA in short-term MetR (and control) mice. **Fig. 1A** outlines the experimental design of all *in-vivo* dietary experiments. **Fig. 1B** outlines the focal stenosis creation, resulting in arterial intimal hyperplasia and remodeling along the RCCA. At day 28 post-op, the RCCA was harvested and processed for histology (**Fig. 1C**). Interestingly, mice preconditioned with MetR had no detectable arterial intimal hyperplasia at POD28, while control-fed mice displayed increased I/M area ratios at 400µM post-stenosis (**Fig. 1D**).

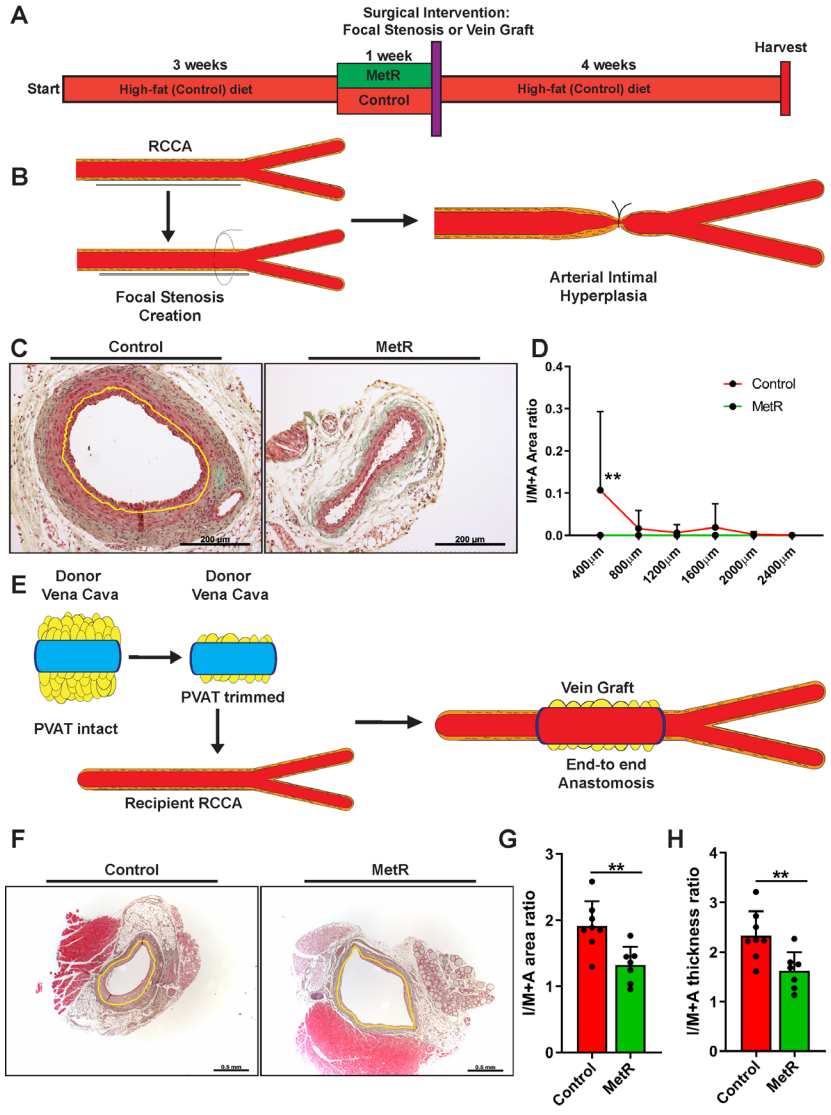


Figure 1. Short-term MetR improves both arterial and vein graft revascularization strategies. A: schematic of dietary intervention. **B:** focal stenosis creation. **C**: RCCA at POD28 after Masson-trichome staining. Yellow line indicates internal elastic lamina lining. Scale bars = 200µm. **D:** I/M area ratio at POD28, via Two-way ANOVA with Sidak's multiple comparisons test, n=8-9/group. **E:** vein graft surgery procedure, with partial trimming of PVAT from donor vena ceva. **F**: vein grafts at POD28 after Masson-trichome staining. Yellow lining indicates internal elastic lamina. Scale bars = 0.5mm. **G**: I/M+A area ratio with Student's t-test, n=7-8/group. **H**: I/M+A thickness ratio with Student's t-test, n=7-8/group. ** P<0.01

We next explored whether vein graft durability could benefit from MetR preceding bypass surgery. **Fig. 1E** outlines the vein graft surgical procedure, as performed routinely[36], which includes partial stripping of caval vein PVAT to better facilitate anastomosis in the recipient.[38] Caval veins from donor mice where then implanted in recipients (on a corresponding diet) via an end-to-end anastomosis. At POD28, vein grafts were harvested and processed for histology (**Fig. 1F**). After morphometric analysis, pre-operative MetR mice had a significant decrease of 31% and 30.5% in I/M+A area (**Fig. 1G**) and thickness (**Fig. 1H**) ratios respectively.

Taken together, these data show that short-term pre-operative MetR can mitigate both the arterial intimal hyperplastic response and vein graft disease after bypass surgery.

3.2 Protection from Vein Graft Disease via Short-term Methionine Restriction is Perivascular Adipose Tissue Dependent.

We next sought to better understand the mechanism by which MetR protects against IH. Considering the potential of perivascular fat to modify surgical outcome[10, 11], together with the published effects of MetR on different adipose depots[30], we hypothesized that protection from VGD by short-term MetR is dependent on modulation of local PVAT phenotype. **Fig. 2A** depicts the experimental design to test this, with the donor vena cava either completely stripped of its PVAT ("No PVAT") or with PVAT left intact ("PVAT"). The vena cava is then transplanted into a recipient on a corresponding diet (Control or MetR), creating a vein graft with or without PVAT (**Fig. 2A-B**) or completely intact (**Fig. 2C-D**). At POD28, grafts were harvested and processed for histology (**Fig. 2E**). Histomorphometric analysis revealed a PVAT-dependent protection from vein graft disease by MetR, as demonstrated by a significant decrease in I/M+A area ratios (53.3% ,**Fig. 2F**), and I/M+A thickness ratios (53.5%, **Fig. 2G**) compared to Control + PVAT; while there was no difference between diet groups without PVAT. Furthermore, two-way ANOVA analysis established a signification diet-PVAT interaction in both vein graft layer thickness and area ratios (**Fig. 2H-I**). MetR + PVAT vein grafts trended towards a smaller intimal area (**Fig. S2A**) but had no change in intimal thickness (**Fig. S2B**). At POD28, lumen area was decreased in MetR + PVAT mice (**Fig. S2C)**, which was confirmed by ultrasound analysis (**Fig. S2D**). Although this also revealed that MetR vein grafts appeared to decrease in lumen diameter at a lesser rate between POD14 and 28 (**Fig. S2D**). The favorable morphology of MetR + PVAT vein grafts was mainly driven by an increase in M+A thickness, (37%, **Fig. 2G**) and area (27.1%, **Fig. 2H**).

Table 1. Percentage of variation between groups that can be explained by diet, PVAT or a diet-PVAT interaction. Two-way ANOVA with Tukey's multiple comparison test on histomorphometric parameters in **Figure 2** & **Fig. S2.** n=11-13/group.

Histomorphometric parameter	Source of Variation	% of total variation	P-value	P-value summary
	Interaction	9.146	0.0146	*
I/M+A area ratio	PVAT +/-	0.006033	0.9482	ns
(Fig. 2F)	Diet	31.16	<0.0001	****
	Interaction	7.538	0.0324	*
I/M+A thickness ratio	PVAT +/-	0.001153	0.9783	ns
(Fig. 2G)	Diet	24.40	0.0003	***
	Interaction	4.274	0.1451	ns
Intimal area	PVAT +/-	0.3439	0.6760	ns
(Fig. S2A)	Diet	10.96	0.0219	*
	Interaction	2.284	0.2734	ns
M+A area	PVAT +/-	0.4127	0.6397	ns
(Fig. 2I)	Diet	16.50	0.0047	**
	Interaction	2.507	0.2736	ns
Intimal thickness	PVAT +/-	1.591	0.3819	ns
(Fig. S2B)	Diet	6.780	0.0751	ns
	Interaction	8.223	0.0159	*
M+A thickness	PVAT +/-	1.254	0.3325	ns
(Fig. 2H)	Diet	35.61	<0.0001	****
	Interaction	6.514	0.0719	ns
Lumen area	PVAT +/-	0.2124	0.7407	ns
(Fig. S2C)	Diet	10.26	0.0253	*

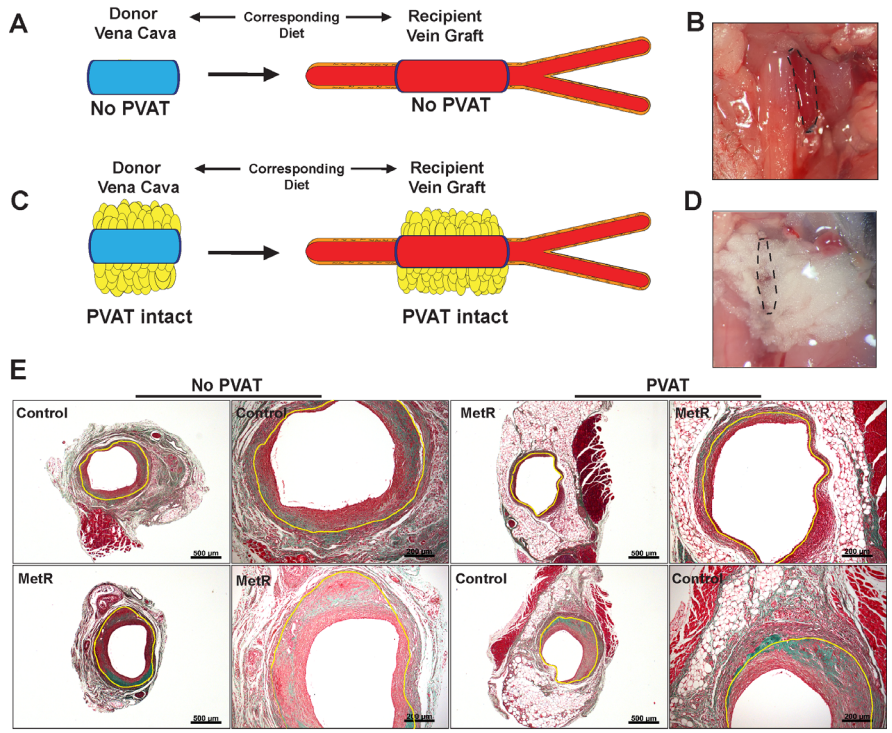


Figure 2. **Protection from Vein Graft Disease via Short-term Methionine Restriction is Perivascular Adipose Tissue Dependent. A-D:** schematic and in-situ images of vena cava/vein graft +- PVAT. **A**: stripping of vena cava PVAT results in vein graft lacking PVAT (**A-B**). **C**: vena cava with PVAT and consecutive vein graft with PVAT intact (**C-D**). **E**: Images of vein grafts at POD28 after Masson-trichome staining. Control-fed and MetR, no PVAT or PVAT. Scale bars 200μm or 500μm as indicated. **F-G**: histomorphometric analysis of POD28 vein grafts. **F**: I/M+A area ratio. **G**: I/M+A thickness ratio. **H:** M+A thickness. **F**: M+A area. All statistical testing was done via two-way ANOVA with Turkey's multiple comparisons test unless otherwise indicated, n=11-13/group. * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001

All data on the interaction between diet and PVAT on these histomorphometric parameters is summarized in **Table 1.** In conclusion, maximal protective effects of MetR on vein grafts were dependent on the interaction between PVAT surrounding the graft and MetR.

3.3 Favorable Vein Graft Wall Composition Seen in MetR Mice is Also Perivascular Adipose Tissue Dependent.

Because favorable vein graft adaptations upon MetR were mainly driven by alterations in outward layer (M+A) remodeling, we next examined how the diet-PVAT interaction impacted the cellular composition of the vein graft wall at POD28. Specifically in the MetR + PVAT group, reduced M_1/M_2-ratios were observed both in the graft wall (**Fig. 3A**) and PVAT (**Fig. 3B**). This favorable M_1/M_2 ratio present in both VG layers was driven by a significant interaction between MetR and the presence of PVAT (**Fig. 3A**, **Table 2)**. The observed differences in M_1/M_2-ratios could be explained by increased polarization towards the M_2-phenotype, both in the intimal/M+A layers (**Fig. 3C-D**) and in MetR PVAT (**Fig. S3A**), while there was a limited decrease in M_1-polarization in the vein graft wall (**Fig. 3C, 3E**) and PVAT (**Fig. S3B)**. Preconditioning with MetR yielded no difference in total M_θ macrophages in the vein graft wall (**Fig. S3C**), nor in surrounding PVAT (**Fig. S3D**). This increase in intimal M_2-macrophages together with an apparent small decrease in M_1-polarized macrophages in the MetR + PVAT cohort could also be linked to the underlying interplay between diet and PVAT (**Fig. 3D-E, Table 2**).

Also at POD28, percentage of intimal area and whole VG occupied by VSMC was dependent on the interaction between diet-PVAT (**Fig. 3F**, **Table 3**), but no significant difference in directionality could be detected between groups (**Fig. 3F**). The absolute number of VSMC per mm^2 present was comparably dependent on a combination of diet and PVAT, but this effect was not strong enough to display significant inter-group differences (**Fig. S3E**). There was no detectable change in proliferating VSMC at POD28 (**Fig. 3H**), nor did extra-cellular matrix analysis reveal a change in collagen deposition (**Fig. S3F**).

Together these data indicate that the interaction between MetR and PVAT prior to transplantation yielded a favorable remodeling phenotype of the graft at POD28, via increased M_2-macrophage polarization, correlating with a beneficial fibroproliferative response. Since mid- and long-term vein graft failure can be

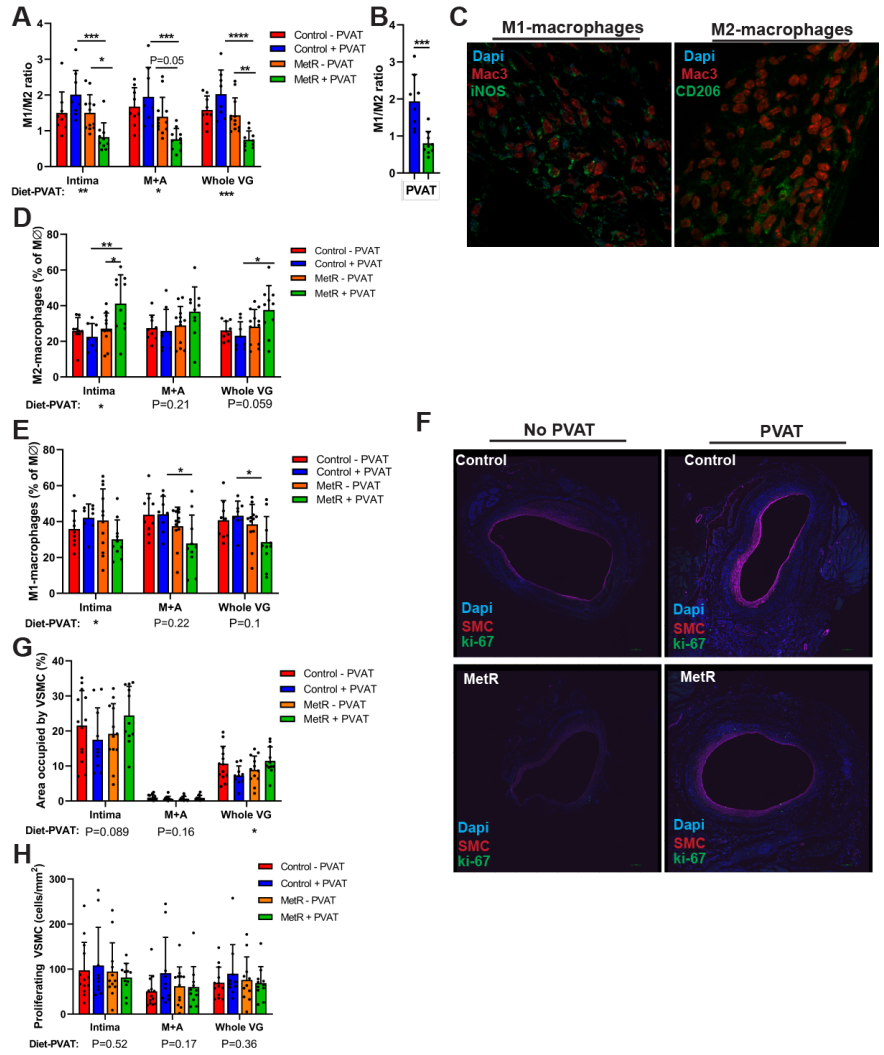


Figure 3. Favorable Vein Graft Wall Composition Seen in MetR Mice is Also Perivascular Adipose Tissue Dependent. A: M1/M2 ratio per vein graft layer. **B:** M1/M2 ratio in PVAT of MetR and control-fed mice. **C:** immunohistochemical staining for M1- and M2-macrophages. **D**: M2-macrophages as a percentage of total MΦ per vein graft layer. **E**: M1-macrophages as a percentage of total MΦ per vein graft layer. **F**: immunohistochemical staining for SMC-α and Ki-67. Scale bars = **G:** area occupied by VSMC per vein graft layer. **H:** proliferating VSMC (SMC-α + Ki-67 double positive cells) per mm^2 per vein graft layer. All statistical testing was done via two-way ANOVA with Tukey's multiple comparisons test, unless otherwise indicated, n=10-13/group. * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001.

Table 2. Percentage of variation between groups that can be explained by diet, PVAT or a diet-PVAT interaction. Two-way ANOVA with Tukey's multiple comparison test on M1/M2 ratio and M2-macrophages (left), M1-macrophages and MΦ macrophages (right). in **Figure 3** & **Fig. S3.** n=10-13/group.

Parameter	Source of Variation	% of total variation	P-value	P-value summary	Parameter	Source of Variation	% of total variation	P-value	P-value summary
M1/M2 ratio	Interaction	20.38	0.0016	**	M1-macrophages	Interaction	10.33	0.0489	*
Intima	PVAT +/-	0.3913	0.6384	ns	Intima	PVAT +/-	0.6856	0.6025	ns
(Fig. 3A)	Diet	20.11	0.0017	**	(Fig. 3E)	Diet	1.899	0.3876	ns
M1/M2 ratio	Interaction	10.70	0.018	*	M1-macrophages	Interaction	3.346	0.2238	ns
M+A	PVAT +/-	1.663	0.3345	ns	M+A	PVAT +/-	2.986	0.2500	ns
(Fig. 3A)	Diet	28.19	0.0003	***	(Fig. 3E)	Diet	17.65	0.0074	**
M/M2 ratio	Interaction	19.86	0.0006	***	M1-macrophages	Interaction	6.346	0.1039	ns
Whole VG	PVAT +/-	0.9584	0.4160	ns	Whole VG	PVAT +/-	2.260	0.3259	ns
(Fig. 3A)	Diet	31.89	<0.0001	****	(Fig. 3E)	Diet	11.99	0.0278	*
M2-macrophages	Interaction	12.01	0.0173	*	MΦ-macrophages	Interaction	20.02	0.0054	**
intima	PVAT +/-	4.668	0.1284	ns	intima	PVAT +/-	0.1291	0.8133	ns
(Fig. 3D)	Diet	15.95	0.0068	**	(Fig. S3F)	Diet	0.00009889	0.9948	ns
M2-macrophages	Interaction	4.024	0.2121	ns	MΦ-macrophages	Interaction	0.8676	0.5669	ns
M+A	PVAT +/-	1.793	0.4019	ns	M+A	PVAT +/-	0.2800	0.7446	ns
(Fig. 3D)	Diet	7.059	0.1012	ns	(Fig. S3F)	Diet	8.404	0.0806	ns
M2-Macrophages	Interaction	8.175	0.0594	ns	MΦ-macrophages	Interaction	0.4932	0.6300	ns
Whole VG	PVAT +/-	2.186	0.3205	ns	Whole VG	PVAT +/-	14.91	0.0113	*
(Fig. 3D)	Diet	14.73	0.0130	*	(Fig. S3F)	Diet	9.798	0.0372	*

traced back to temporal processes and adaptations immediately after graft implantation[9], we next evaluated baseline effects of MetR on PVAT and how this modulated vein graft PVAT during early remodeling.

3.4 Short-term MetR Modulates Caval Vein Perivascular Adipose Tissue Towards an Arterial-like Phenotype.

To delineate the effects of MetR on venous PVAT and to assess how MetR alters the response in PVAT to surgical injury (vein graft surgery), we performed transcriptomic analysis on caval vein PVAT and on PVAT from POD1 vein grafts (**Fig. S4A**). These baseline and early time points after surgery, when MetR diets were also halted, were chosen to test the hypothesis that the diet/adipose interaction was present at baseline, and capable of modulating the response during early vein graft remodeling. Thoracic aorta PVAT from control-fed and MetR mice was also sequenced to investigate arterial PVAT, which could not be harvested from RCCA due to technical limitations (**Fig. S4A**).

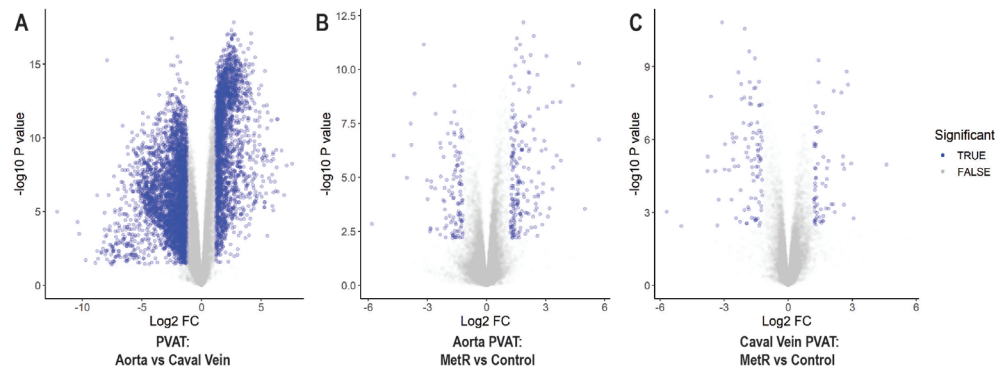
At baseline, venous (caval vein) PVAT from control-fed mice was distinct from arterial (aorta) PVAT (4568 genes, **Fig. 4A, D)**. Pathway analysis of these differentially expressed genes in arterial PVAT revealed a brown-adipose tissue like phenotype, with increased *thermogenesis* and *AMPK signaling* (**Fig. 4E)**. In arterial PVAT, MetR modified 1316 gene-transcripts (**Fig. 4B, D**) while further inducing *AMPK signaling* and *thermogenesis* pathways (**Fig. 4E**) compared to control-fed mice. In caval vein PVAT, MetR modified 811 different transcripts (**Fig. 4C**), including activation of *AMPK signaling* and *focal adhesion* pathways (**Fig. 4E**). Together, these data suggest that perivascular fat resembles BAT in terms of energy consumption rather than energy storage, and that MetR promotes this phenotype in both arterial and venous PVAT via canonical downstream activation of thermogenesis genes via AMPK[39] (**Fig. 4E**).

Analysis of PVAT at POD1 revealed vein graft surgery as a much larger modulator of gene expression than diet, with clear distinctions between baseline and POD1 in both diet groups, including 7492 and XXX? transcripts that were differentially regulated compared to baseline in control and MR groups, respectively (**Fig. 4F**). Despite the larger global effect of surgery, there were multiple differentially regulated genes as a function of diet on POD1 (**Fig. 4G**). Multiple pathways involved in PVAT energy-metabolism (AMPK signaling, fatty-acid biosynthesis) were differently regulated between diet groups in response to the surgery (**Fig. S4A-B)**. Next to regulation of energy storage and consumption genes, several pathways involved in the immune response showed an alternate response

between control and MetR, including hematopoietic linage (**Fig. S4C)**, primary immunodeficiency (**Fig. S4D**), B-cell receptor signaling (**Fig. S4E)** and toll like receptor signaling (**Fig. 4G**). Analysis of individual gene transcripts within these pathways revealed several differentially regulated transcripts in vein graft PVAT at POD1 as a result of MetR preconditioning. Among others, we observed a dampening of Tlr4 transcript expression (**Fig. S4H-I**) together with a robust decrease in transcripts for its endogenous ligand[40] tenascin-C (**Fig. 4I**) in the MetR group. Interestingly, the pro-atherosclerotic and pro-restenosis enzyme lysyl-oxidase[41] (LOX) was also strongly downregulated in MetR PVAT compared to control-fed at POD1 (**Fig. 4J**).

Table 3. Percentage of variation between groups that can be explained by diet, PVAT or a diet-PVAT interaction. Two-way ANOVA with Tukey's multiple comparison test on either % of vein graft layers occupied by VSMC, VSMC/mm^2, VSMC colocalizing with Ki-67 or % of vein graft layers occupied by collagen. As depicted in graphs in **Figure 3** & **Fig. S3.** n=10-13/group.

Parameter	Source of Variation	% of total variation	P-value	P-value summary	Parameter	Source of Variation	% of total variation	P-value	P-value summary
% VSMC of	Interaction	6.622	0.0897	ns	VSMC/mm^2	Interaction	4.246	0.1832	ns
Intima (Fig. 3G)	PVAT +/- Diet	0.1041 1.583	0.8286 0.4005	ns ns	Intima (Fig. S3E)	PVAT +/- Diet	0.008998 1.033	0.9506 0.5080	ns ns
% VSMC of	Interaction	4.430	0.1693	ns	VSMC/mm^2	Interaction	6.810	0.0860	ns
M+A	PVAT +/-	0.1700	0.7855	ns	M+A	PVAT +/-	1.037	0.4962	ns
(Fig. 3G)	Diet	0.1062	0.8296	ns	(Fig. S3E)	Diet	1.278	0.4504	ns
% VSMC of	Interaction	13.01	0.0153	*	VSMC/mm^2	Interaction	10.20	0.0355	*
Whole VG	PVAT +/-	0.2532	0.7262	ns	Whole VG	PVAT +/-	1.095	0.4802	ns
(Fig. 3G)	Diet	2.068	0.3194	ns	(Fig. S3E)	Diet	0.4385	0.6545	ns
VSMC+ Ki-67	Interaction	0.9706	0.5219	ns	% collagen of	Interaction	2.705	0.2627	ns
intima	PVAT +/-	0.01213	0.9428	ns	Intima	PVAT +/-	1.738	0.3681	ns
(Fig. 3H)	Diet	1.456	0.4334	ns	(Fig. S3E)	Diet	3.099	0.2311	ns
VSMC+ Ki-67	Interaction	4.180	0.1743	ns	% collagen of	Interaction	0.05245	0.8773	ns
M+A	PVAT +/-	3.468	0.2151	ns	M+A	PVAT +/-	1.229	0.4562	ns
(Fig. 3H)	Diet	0.8681	0.5323	ns	(Fig. S3E)	Diet	2.802	0.2625	ns
VSMC+ Ki-67	Interaction	2.150	0.3458	ns	% collagen of	Interaction	0.7912	0.5427	ns
Whole VG	PVAT +/-	0.5066	0.6458	ns	Whole VG	PVAT +/-	2.748	0.2591	ns
(Fig. 3H)	Diet	0.6122	0.6135	ns	(Fig. S3E)	Diet	3.815	0.1848	ns



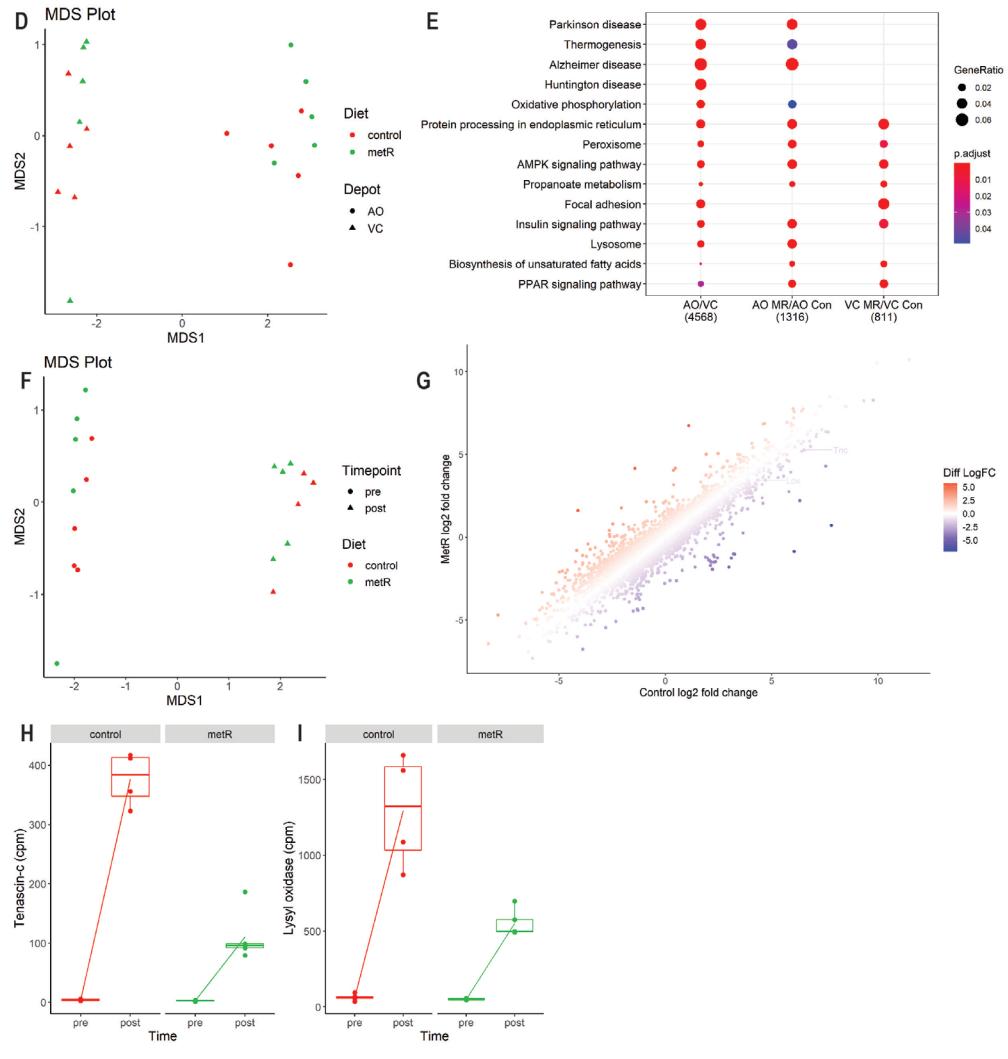


Figure 4. Short-term MetR Modulates Caval Vein Perivascular Adipose Tissue Towards an Arterial-like Phenotype and Dampens Post-op Inflammation A: fold change in transcript expression in control-fed aorta PVAT versus control-fed caval vein PVAT. **B**: fold change in transcript expression in aorta PVAT of MetR versus control-fed mice. **C**: fold change in transcript expression in caval vein PVAT of MetR versus control-fed mice. **D**: principal component analysis of aorta and caval vein PVAT of control-fed and MetR mice. **E**: pathway analysis of aorta versus caval vein PVAT of control-fed mice (AO/VC, first column), aorta PVAT MetR versus control-fed (AO MR/AO Con, second column) and caval vein PVAT MetR versus control-fed (VC MR/ VC Con, third column). **F**: principal component analysis of control-fed and MetR PVAT from caval vein and POD1 vein grafts. **G**: fold change in transcript expression between caval vein PVAT and vein graft PVAT at POD1 for both control-fed and MetR mice. **H**: tenascin-c transcript expression (in counts per million) in control-fed and MetR mice caval vein and vein graft PVAT. **I**: lysyl oxidase transcript expression (in counts per million) in control-fed and MetR mice caval vein and vein graft PVAT.

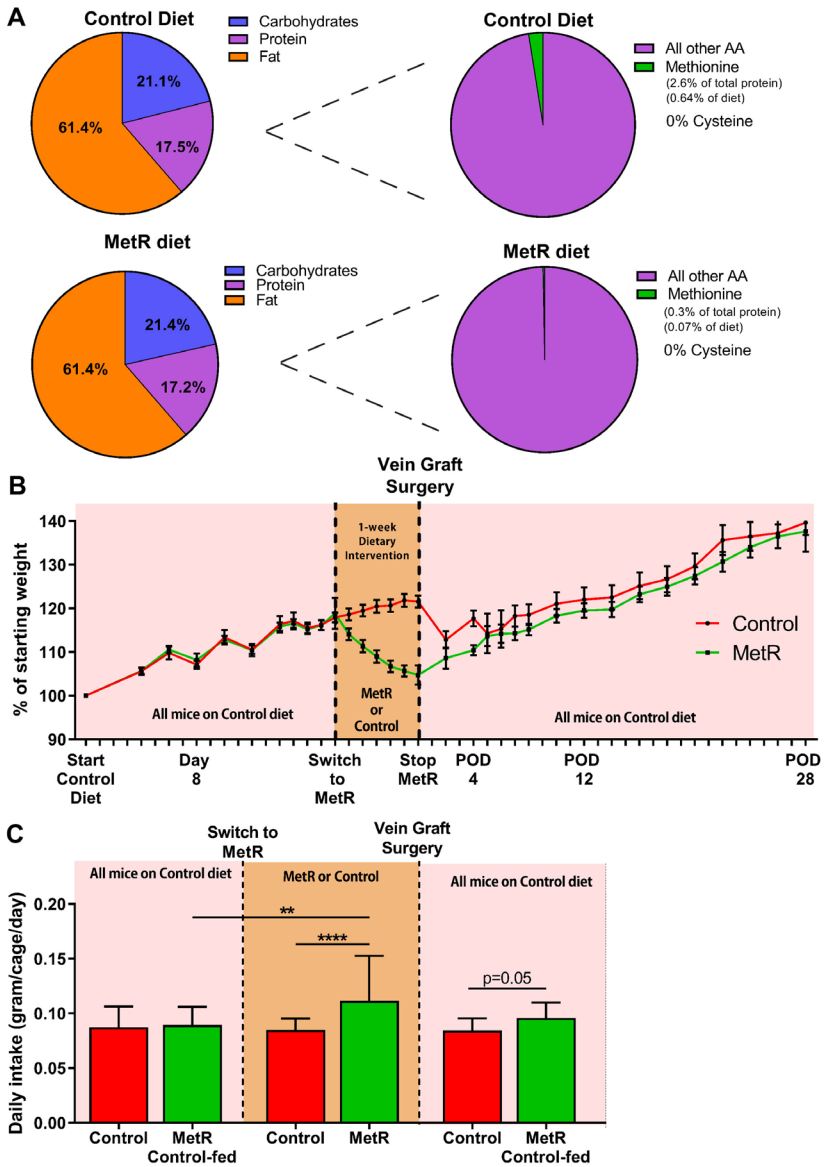
4. Discussion.

In this current study, we tested the potential of short-term dietary restriction of the sulfur amino-acids methionine and cysteine, in cardiovascular surgery preclinical models. This MetR intervention, which constitutes an isocaloric diet with adequate levels of all macronutrients, protected from arterial IH in a focal stenosis model, and improved vein graft adaptation to an arterial circulation in a model of bypass surgery. Specifically, in bypass surgery, MetR further improved graft remodeling when the donor caval vein was implanted with PVAT intact. Control-fed mice who received a caval vein with intact PVAT had exacerbated VGD at POD28, pointing towards a diet-induced reversal of PVAT phenotype in MetR mice compared to control diet. There was no significant histomorphometric difference detectable between diet groups when caval veins were stripped of PVAT before anastomosis creation, suggestive of a PVAT-dependent protection from VGD yielded by MetR.

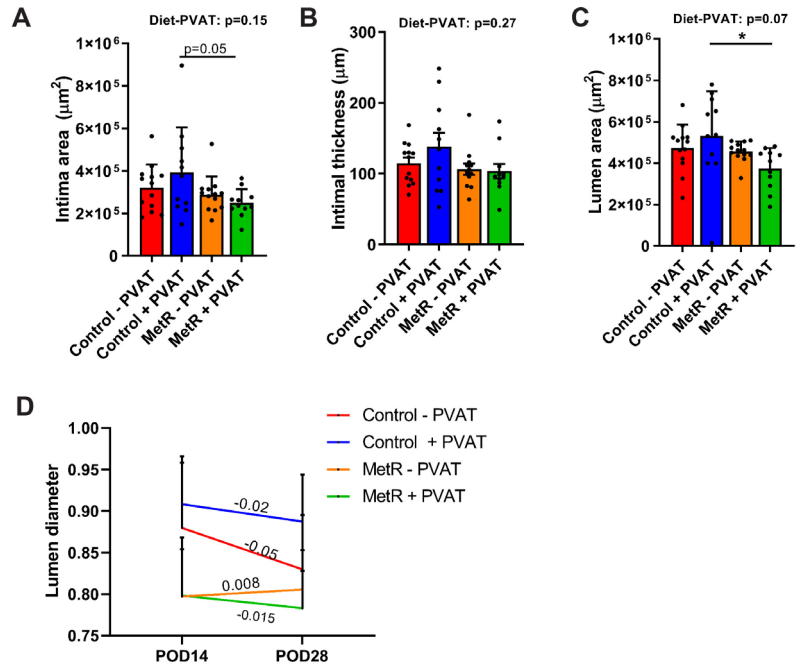
Whether protection from VGD is conferred via MetR in the donor or recipient mouse, or whether this concerns an interplay between local PVAT phenotype and systemic (recipient) effects of diet remains unclear. Recently we found that a short-term reduction of total proteins before vein graft surgery protects from VGD.[27] There, we also tested whether protection originated from protein restriction in either the donor or the recipient and found that recipient-only restriction was adequate for protection and that this was enhanced when utilizing both donor and recipients on protein restriction diets. Simultaneously, donor-only restriction was not, which appears to contradict our current findings on the importance of donor PVAT. However, in that study and in our initial MetR vein graft study (**Fig. 1E-H**), we partially stripped the caval vein of its PVAT. This could account for both the apparent inability of donor-only restriction to yield protection in our previous study, as well as the difference in effect-size after MetR between **Fig. 1E-H** and **Fig. 2F-G**.

Our in-depth analysis and side-by-side comparison of arterial and venous PVAT revealed a distinct transcriptomic profile between vein and artery PVAT, with venous PVAT resembling white/beige adipose tissue.[42] Arterial PVAT closely resembled BAT, as seen by the presence of several genes involved in thermogenesis and in accordance with previous studies.[43] Preconditioning with MetR further upregulated thermogenesis gene transcripts, together with an increase in upstream AMPK-signaling. In venous PVAT, although thermogenesis was not increased, AMPK signaling was up, suggestive of browning of this white/beige adipose tissue depot.

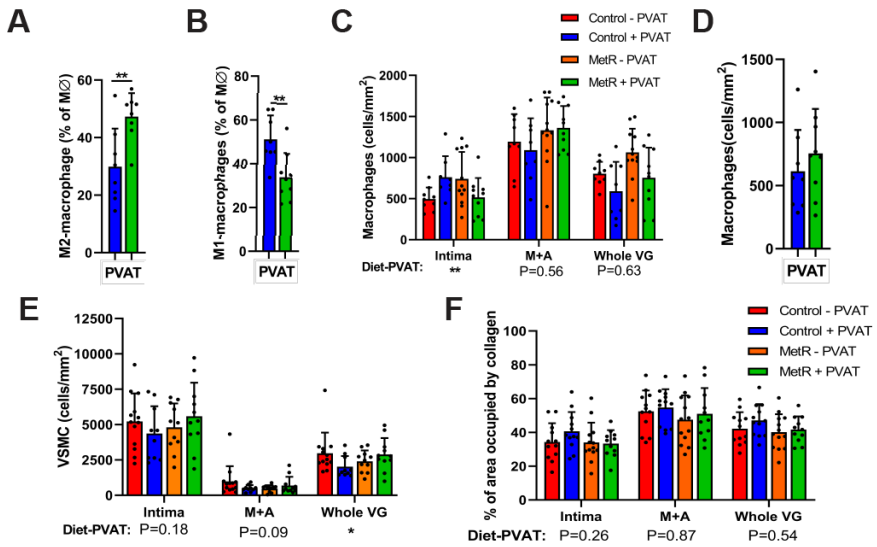
Here we show that short-term MetR attenuates VGD, and that this effect is dependent on intact vein PVAT during implantation. MetR activated both venous and arterial PVAT AMPK signaling and consequently browning in arterial and possibly venous PVAT. Suggestive of caval vein PVAT with a BAT/arterial PVAT signature after MetR, which could explain (part) of the protection from VGD after MetR. Follow-up studies should determine the cell types involved in protection from VGD by MetR. Furthermore, donor vs recipient and immune-cell labeling could determine the mechanistic interplay between MetR, donor PVAT and the recipients local and systemic characteristics that together attenuate VGD.



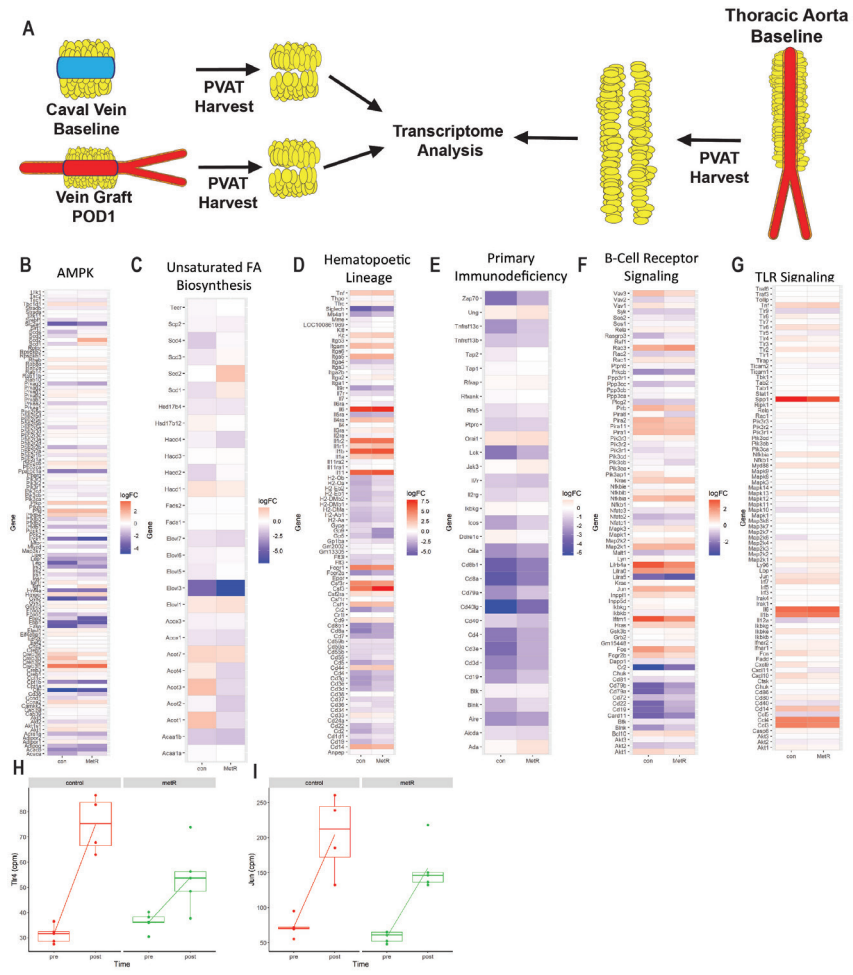
Supplemental figure 1. Methionine restriction diet composition and metabolic response. A: Macronutrient composition of control and methionine restricted diet. Both diets contain 0% cysteine and 60% fat. **B:** representative graph of percentage of starting weight that was lost/gained during the study. **C**: representative bar graph of daily food-intake during study in both groups. ** P<0.01, **** P<0.0001. All statistical testing was done via two-way ANOVA with Turkey's multiple comparisons test, unless otherwise indicated.



Supplemental figure 2. Intimal area/thickness and lumen diameters at POD28. A-C: Histomorphometric analysis of vein grafts at POD28. **A**: Intimal area. **B**: Intimal thickness. **C:** Lumen area. **D**: Lumen diameter ultrasound measurements at POD14 and POD28, with rate of lumen increase/decrease. All statistical testing was done via two-way ANOVA with Tukey's multiple comparison's test unless otherwise indicated, n=11-13/group. * P<0.05



Supplemental figure 3. VSMC density, collagen content and MΦ macrophages in Vein Grafts and PVAT at POD28. A: M_2-macrophage count in PVAT of control-fed and MetR mice, in cells/mm^2. **B**: M_1-macrophage count in PVAT of control-fed and MetR mice, in cells/mm^2. C: MΦ-macrophages per vein graft layer in cells/mm^2. **D**: MΦ-macrophage count in PVAT of control-fed and MetR mice, in cells/mm^2. **E**: VSMC density per vein graft layer in cells/mm^2. **F**: percentage of vein graft layer occupied by collagen. All statistical testing was done via two-way ANOVA with Tukey's multiple comparisons test, unless indicated otherwise, n=10-13/group. ** P<0.01.



Supplemental figure 4. Transcriptome analysis of control-fed and MetR PVAT. A: schematic of caval vein and vein graft POD1 PVAT; and of thoracic aorta PVAT harvest, three separate adipose tissue depots processed for transcriptome analysis simultaneously. **B-G**: gene-patway heatmaps for control and MetR-fed mice, fold change between baseline versus POD1. **B**: AMPK-pathway gene heatmap. **C**: Unsaturated fatty-acid biosynthesis. **D**: Hematopoetic lineage pathway. **E:** Primary Immunodeficiency. **F:** B-cell receptor signaling. **G: F-H**: expression of tlr4 and jun transcipts (in counts per million) in caval vein and vein graft PVAT from control-fed and MetR mice.

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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**).

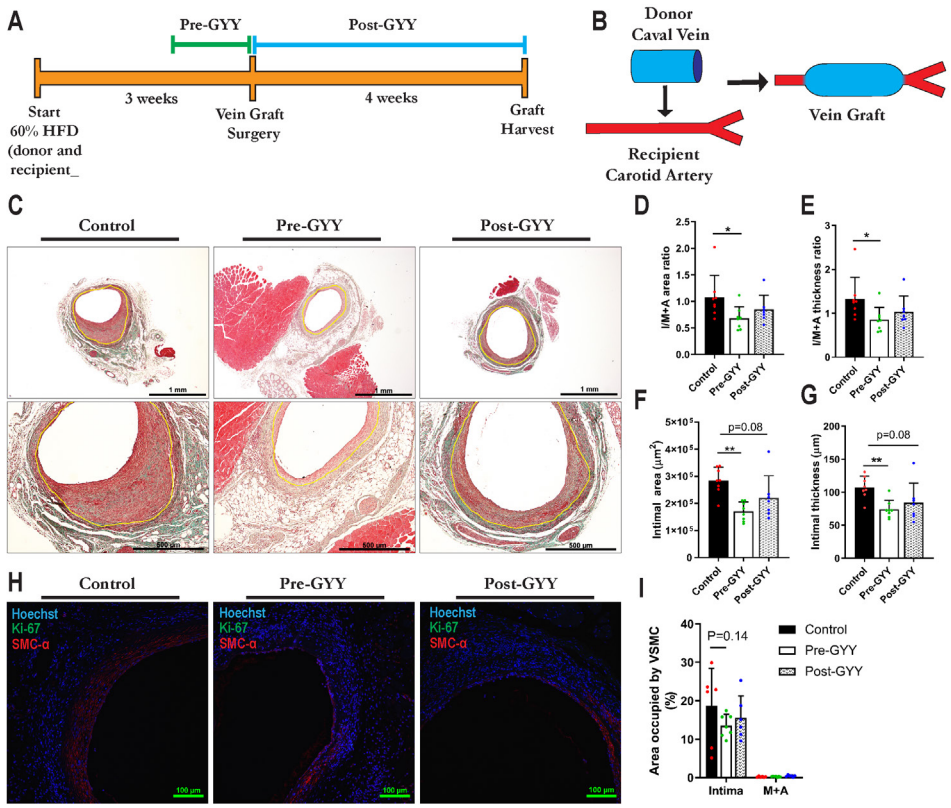


Figure 1. Systemic therapy with the H_2S donor GYY limits vein graft disease. **A:** Schematic depiction of the vein graft procedure: end-to-end anastomosis of a donor mouse caval vein into a recipient mouse right common carotid artery. **B:** Experimental design: 60% high-fat diet (HFD), 1 week of pre-conditioning (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**).

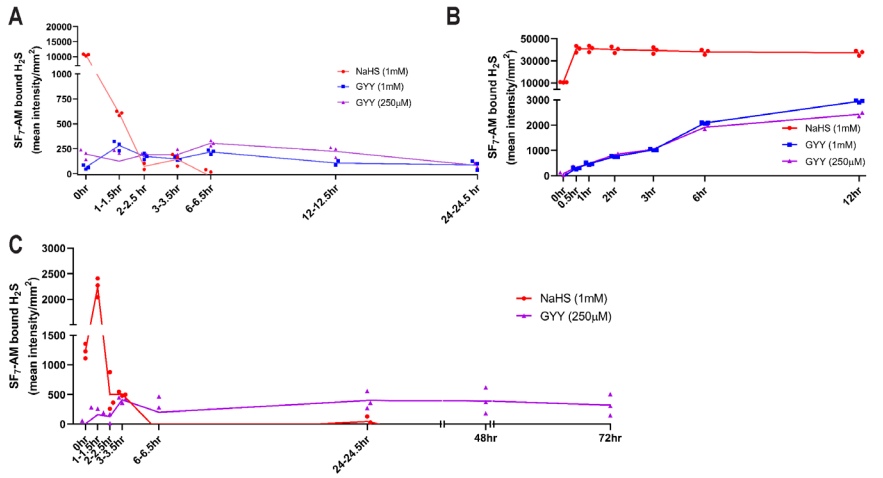


Figure 2. Development of a locally applicable pluronic gel that ensures extended H_2S release both ex-vivo and in-vivo

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.

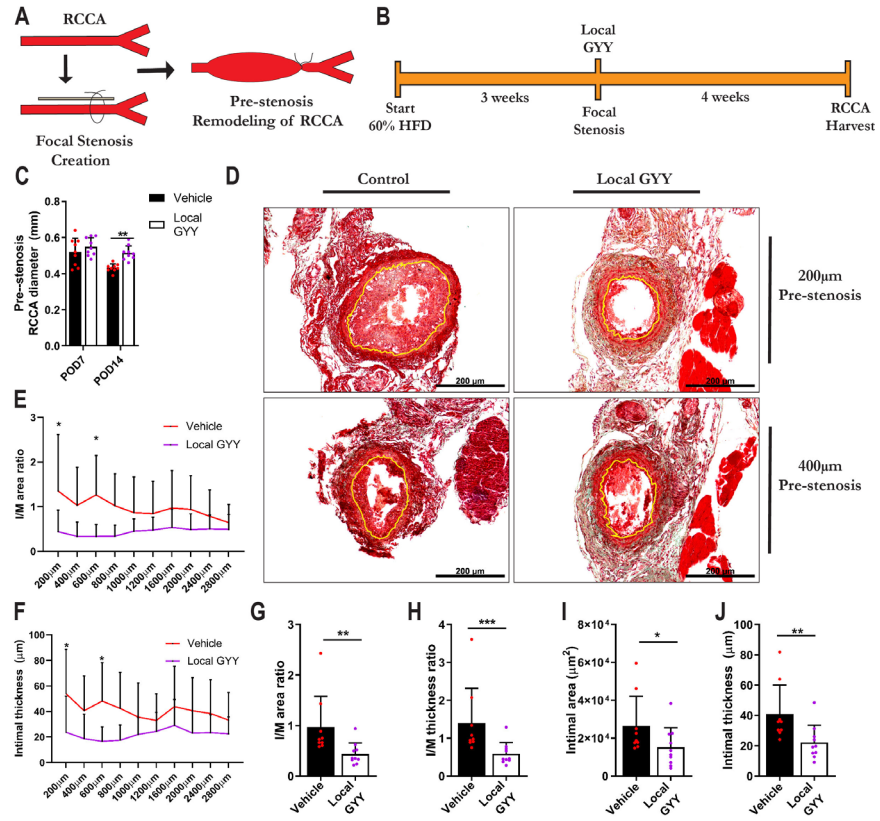


Figure 3. Local application of the H_2S donor GYY mitigates injury induced arterial intimal hyperplasia.

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

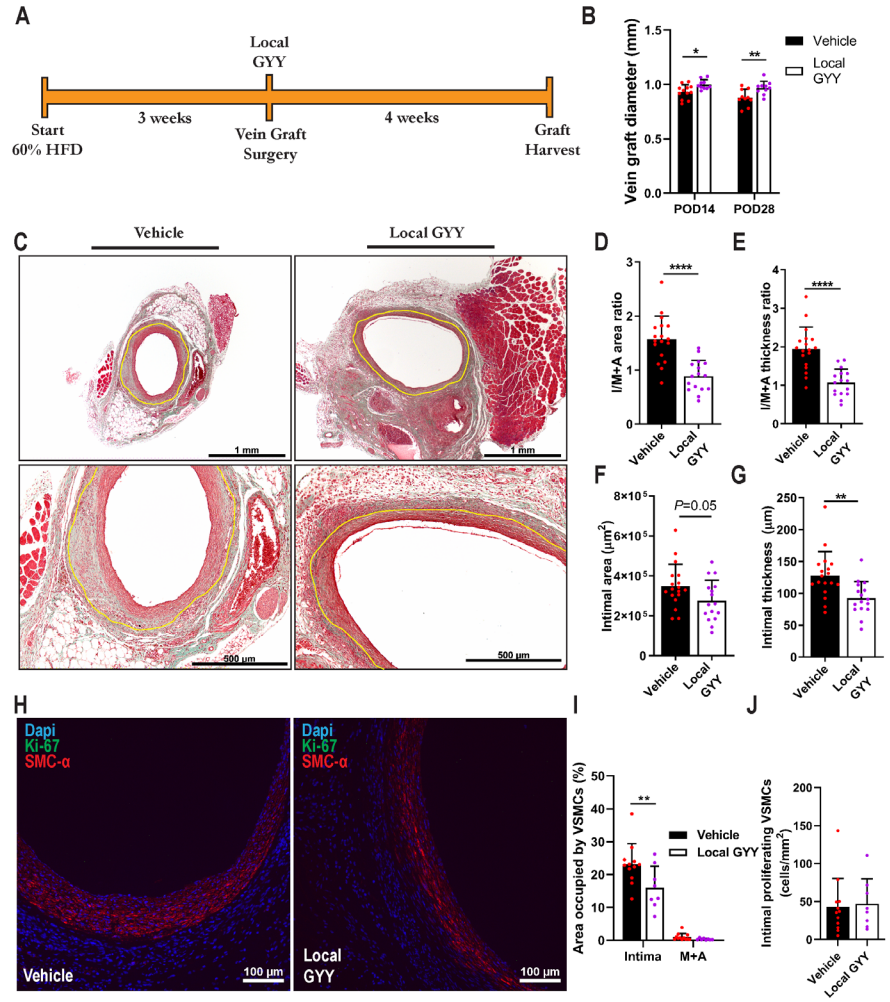


Figure 4. **Periprocedural local H_2S therapy protects from vein graft disease by attenuating intimal VSMC migration.**

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]

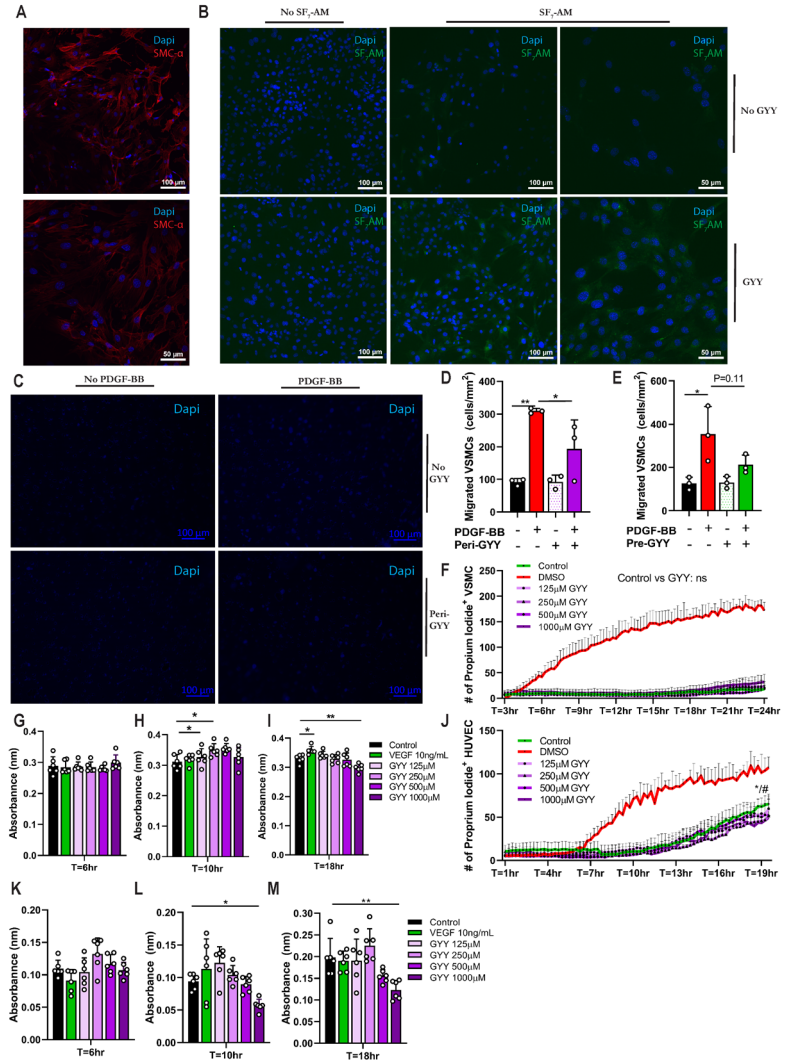


Figure 5. Periprocedural H_2S therapy limits VSMC migration in-vitro via

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.

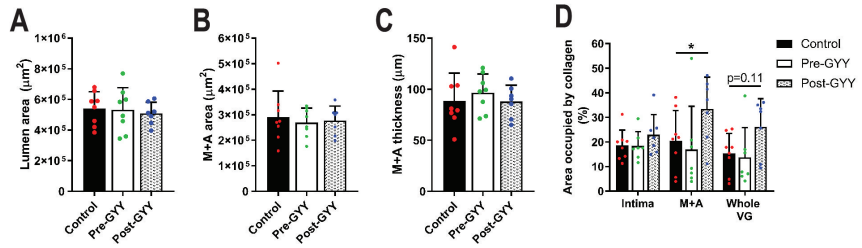
5. Funding.

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6. Acknowledgements.

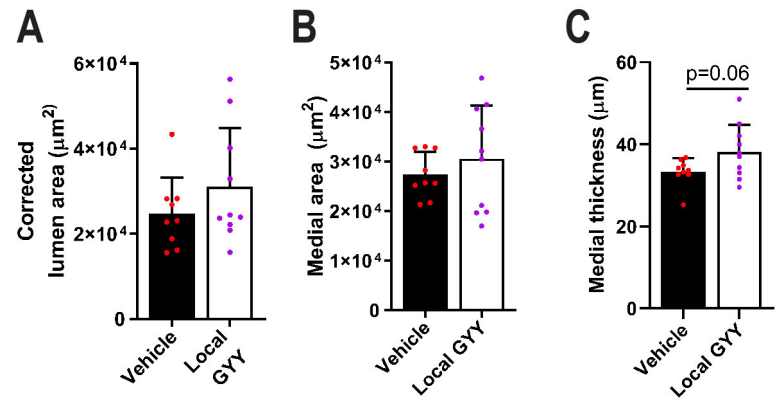
P.K. provided funding, conceived of experimental designs, performed experiments and wrote the manuscript. M.T. performed surgeries, processed vein grafts and analyzed histology. M.R.V. conceived of experimental designs, performed surgeries and advised with P.H.Q. on analysis of vein grafts. K.T., M.R.M. 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.



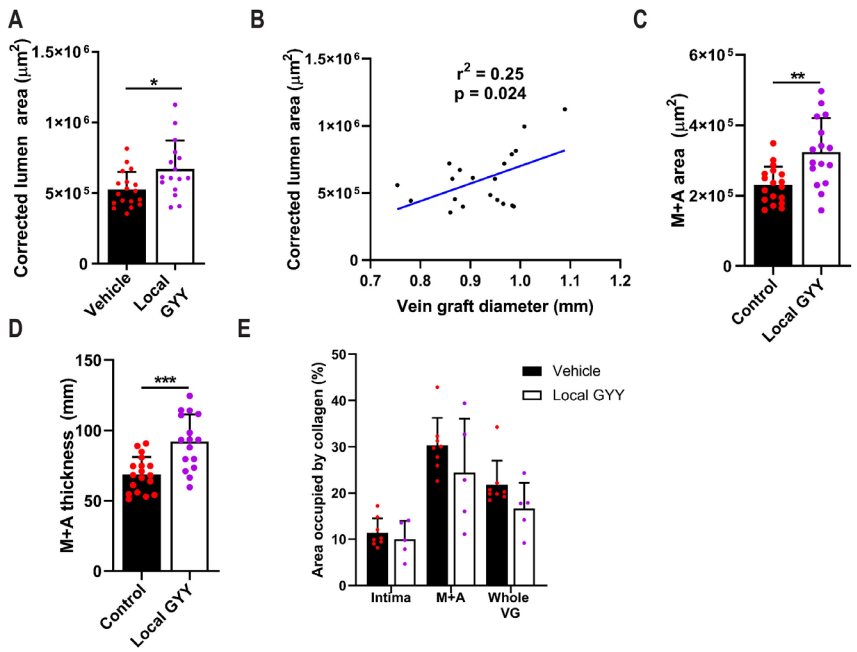
Supplemental 1, related to Figure 1.

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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Chapter 6.

Insights from A Short-Term Protein-Calorie Restriction Exploratory Trial in Elective Carotid Endarterectomy Patients.

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Abstract.

Background.

Open vascular surgery interventions are not infrequently hampered by complication rates and durability. Preclinical surgical models show promising beneficial effects in modulating the host response to surgical injury via short-term dietary preconditioning. Here we explore short-term protein-calorie-restriction preconditioning in patients undergoing elective carotid endarterectomy to understand subject participation dynamics and practicalities of robust research approaches around nutritional/surgical interventions.

Methods.

We designed a pilot prospective, multicenter-randomized-controlled study in patients undergoing carotid endarterectomy. After a 3:2 randomization to a 3-day pre-operative protein-calorie-restriction regimen (30% calorie/70% protein restriction) or *ad libitum* group, blood, clinical parameters and stool samples were collected at baseline, pre-op, post-op days 1&30. Subcutaneous and perivascular adipose tissue was harvested periprocedurally. Samples were analyzed for standard chemistries and cell counts, adipokines and cytokines. Bacterial DNA isolation and 16S rRNA sequencing was performed on stool samples and bacterial species relative abundance was measured.

Results.

51 patients were screened, 9 patients consented to the study, 5 were randomized, 4 completed the trial. The main reason for non-consent was a 3-day in-hospital stay. All 4 participants were randomized to the protein-calorie-restriction group, underwent successful endarterectomy, reported no compliance difficulties nor were there adverse events. Stool analysis trended towards increased abundance of the sulfide-producing bacterial species *Bilophila wadsworthia* after dietary intervention (p=0.08).

Conclusions.

While carotid endarterectomy patients held low enthusiasm for a 3-day pre-operative inpatient stay, there were no adverse effects in this small cohort. Multi-disciplinary longitudinal research processes were successfully executed throughout the nutritional/surgical intervention. Future translational endeavors into dietary preconditioning of vascular surgery patients should focus on outpatient approaches.

1. Introduction.

With the significant burden of cardiovascular disease,[1] and consequently necessary cardiovascular surgical operations with subsequent revascularization attempts and the inevitable complications (durability, wound healing, peri-procedural cardiac events, etc.) associated with said procedures (especially open operations), there remains a high demand for cost-effective measures to enhance the value of cardiovascular interventions. Manipulation of the mammalian response to injury stands as a logical strategy to meet this need.

In recent years, modulation of the host surgical injury response via short-term dietary restriction has emerged as an intervention with beneficial clinical potential in preclinical rodent models.[2] Dietary restriction serves as an umbrella term for the reduction of total calories, total protein, specific amino-acids or a combination of these elements. Calorie restriction, i.e. reducing calories without altering food composition, can influence the response to surgical trauma[3], protects from ischemia-reperfusion damage in liver and kidney[2, 4] and reduces cerebral pathology and parasite load in a mouse malaria model.[5] Protein restriction, i.e. replacing protein with carbohydrates, attenuates the arterial hyperplastic response to focal injury,[6] limits kidney and liver damage after ischemia reperfusion,[4, 6, 7] is protective in a rat model of focal stroke[8], improves durability after vein graft bypass surgery[9] and improves perioperative glucose homeostasis without negatively affecting wound healing.[10] In the limited scope of renal ischemia-reperfusion injury in which the interaction between protein and calorie restriction has been interrogated, both contributed additionally to protection.[7]

Mechanistically, some of the benefits of these preoperative dietary preconditioning strategies in rodents can be contributed to increased production of hydrogen sulfide.[11] a gaseous vasodilator[12] with anti-inflammatory,[13] cytoprotective,[14, 15] proangiogenic,[16] and anti-atherosclerotic[14, 17] potential in the vascular system. Emerging literature also supports a mechanistic link to microbiome mediated effects.[18-20]

Although some work has been done in humans examining the potential of these preconditioning strategies,[21-24] no studies exist that interrogate the feasibility of short-term dietary interventions in the aged, medically complicated, and often frail vascular surgery patient population. Furthermore, future scientific discovery into the protective mechanisms of short-term pre-surgical dietary

restriction will likely involve such diverse scientific fields such as gaseous signaling molecules, immune-regulation, adipose biology, and the microbiome, making essential the construction of a multi-disciplinary "discovery platform" research team that can efficiently collect and analyze a variety of clinical specimens real-time as patient care progresses through a complex, real-world surgical care system.

Here we report on insights gained from a prospective, randomized, multi-institutional exploratory human study of short-term protein and calorie restriction (PCR) in patients undergoing elective carotid endarterectomy (CEA). We examine the feasibility of clinical research processes in this specific patient population and the scientific practicality of longitudinal multi-disciplinary analyses of the biologic mechanistic links to H_2S biology and beyond. Essentially, we hypothesize that short-term PCR is feasible in elective patients, and that a variety of important biologic endpoints can be confidently assayed in such patients.

2. Methods.

Trial Design and Setting.

This prospective, multicenter, randomized, controlled study was approved by the Partners Human Research Committee institutional review board and registered with ClinicalTrials.gov (Identifier: NCT03303534) to enroll up to 40 subjects at two academic tertiary medical centers: Brigham and Women's Hospital (BWH) and Beth Israel Deaconess Medical Center (BIDMC). Professionally produced, IRB approved, videos were utilized to recruit and educate patients on the trial. Written informed consent was obtained by a physician-investigator for subjects to be admitted for 3 days pre-operatively to inpatient research units at the Center for Clinical Investigation (BWH) and Clinical Research Center (BIDMC) where they arrived NPO and were randomized to a control (*ad libitum)* or PCR diet. Additionally, diabetic patients were evaluated by an endocrinologist during the course of the dietary intervention. Subjects were discharged from the research units on the day of elective CEA, and they underwent typical same day admit processes, and after-surgery care per standard clinical practice. The study included direct and indirect observation for up to 30 days post-operatively. Study assessments at 14 days and 30 days post-operatively occurred at regular outpatient office visits (**Figure 1**). Additional research assessments occurred at 30 days post-operatively

at the Clinical Trials Hub at BWH. This fast-tracked, pilot study took place from September 2017 to May 2018 (the timepoint when charges/rates at the inpatient research units increased to a point that was cost prohibitive for this initiative).

Participants.

Men and women 18 years and older with a clinical indication for elective CEA, as determined by the attending vascular surgeon, were included. Symptomatic patients were eligible on the condition that the time needed for the PCR intervention was deemed safe by the attending vascular surgeon. Excluded were patients with allergies or intolerance to any of the diet ingredients, active infection, pregnancy, malnutrition based on anthropometric measurements and serum albumin of less than the 3.5 g/dL, drug/alcohol dependency or active non-cutaneous cancer treatment with chemotherapy.

Randomization.

Employing a randomized (3:2), parallel design, subjects were assigned to either the supervised PCR diet (Scandi-Shake [any of 4 flavors – vanilla, strawberry, banana cream, and caramel] mixed with almond milk, calculated individually for a total daily volume to achieve 30% caloric restriction and 70% protein restriction, based on an individually calculated daily energy requirement), or continued routine *ad libitum* (AL) feeding on their normal diet. This occurred upon admission to the inpatient research unit.

Blinding.

All non-essential clinical staff were blinded to the study arm, including basic science laboratory research assistants, to ensure unbiased interpretation of clinically obtained specimens. Each subject was assigned a unique subject number.

Data Collection.

Dietary compliance.

The Mifflin St. Jeor equation25 was employed by licensed dieticians to calculate the 24-hour energy needs of the individual participants based on gender, age, height, weight and activity factor. A paper food diary was employed for subjects to record their food intake while enrolled in the study, which was used by dieticians to calculate daily nutritional intake.

Clinical parameters.
Blood pressure, heart rate and weight were recorded on a daily basis during the inpatient section of the study and at the post-op day 14 and 30 follow-up appointment.

Blood draws.
Blood was collected via venipuncture at baseline upon admission 3 days prior to surgery before the start of dietary intervention, the day of surgery (after 3 days of dietary preconditioning), the day after surgery, and at the 30-day post-operative visit. Blood samples were processed by technicians at the Center for Clinical Investigation at BWH and tested by the Laboratory Corporation of American Holdings (LabCorp). At all four timepoints, LabCorp testing consisted of 5.0mL of whole blood collected in an ethylenediaminetetraacetic acid (EDTA) coated tube for a complete blood count with differential and 10.0mL of whole blood in a red top tube (RTT) for a basic metabolic panel, cortisol, insulin growth factor-1, and c-reactive protein. At baseline and pre-operatively, LabCorp testing also included pre-albumin as part of the 10.0mL collected in the RTT. In addition, 8.0mL of whole blood in a RTT was centrifuged (Thermo Forma 5681 3L GP) for 15 minutes at 2500 *g* to be stored as serum at -80°C, ELISA for FGF-21 and Insulin was then performed by study staff.

Adipose tissue biopsy.
During the CEA procedure, the attending surgeon harvested a 2 mm^3 subcutaneous adipose tissue specimen from the subcutaneous fat depot at the incision site. A 2 mm^3 biopsy from the perivascular adipose tissue immediately surrounding the carotid artery was also harvested before start of the endarterectomy. Both specimens were snap frozen in liquid nitrogen and then stored at -80°C.

Stool samples and Bacterial DNA isolation and 16S rRNA sequencing.
Fecal samples from the four patients at various time points were collected to measure longitudinal shifts in the gut microbial community; baseline, day 1 of diet, day 2 of diet, day of surgery, post-operative day 14, and post-operative day 30. A total of 20 fecal samples were collected, immediately snap-frozen, and sent to the Nutritional and Microbial Ecology Lab at Harvard University for storage at -80° C until analysis. Microbial DNA was extracted using the DNeasy PowerSoil Kit (Qiagen, Cat# 12888-100). The V4 region of the 16S rRNA gene was PCR amplified in triplicate using custom barcoded primers (515F and 806Rbc), with sample-specific negative controls. The PCR amplification took place in Bio-Rad T100 Thermocyclers using the following sequence: 94°C for 3 minutes, followed by 35 cycles of 94°C for 45 seconds, 50°C for 30 seconds, and 72°C for 90 seconds, with a final extension at 72°C for 10 minutes.[26] The success of each amplification was confirmed using gel electrophoresis to ensure that an amplicon of the expected size (381 bp) was produced. Amplicons were cleaned with Agencourt AMPure XP beads (Beckman Coulter, Inc., Cat# A63882). Cleaned amplicons were quantified using the Quant-iT PicoGreen dsDNA Assay Kit following manufacturer's instructions (Invitrogen, Cat# P11496), and 80 ng of DNA from each sample was entered into a common pool. The amplicon pool was then purified using the Qiaquick MinElute Kit (Qiagen, Cat# 28004) and separated from the off-target products by gel purification using the QIAquick Gel Extraction Kit (Qiagen, Cat# 28704). The pool was adjusted to 10nM and sequenced 1 x 150 bp on one lane of an Illumina HiSeq rapid flow cell at the Harvard Bauer Core.

Primary patency after carotid endarterectomy.
Patency of the carotid artery was determined by duplex ultrasound at the 30-day visit, as performed by ICAVL certified vascular ultrasound laboratory.

ELISA.
FGF-21(DF2100, R&D systems,) and Insulin (80-INSHUU-E01.1, ALPCO) assays were performed according to the respective manufacturer's protocol. Briefly, serum samples were thawed and resuspended in duplicates on 96-well plates pre-coated with the respective primary antibodies for human FGF-21 and Insulin. On both plates, a titration dilution of standard stock solution of FGF-21 and Insulin was added in duplicates. After a 1-2 hour incubation period at room temperature (RT), samples were washed 6 times with 400µL washing buffer per well (20x, diluted in diH_2O, supplied by the manufacturer). For FGF-21, 200 µL of human FGF-21 conjugate was added per well and the plate incubated for 2 hours at RT, then washed 6 times with 400 µL of washing buffer. 200 µL Substrate Solution was added per well, and the plate incubated for 30 minutes in the dark. After 30 minutes, 50 µL of Stop Solution was added per well and absorbance was measured in a microplate reader (BiotTek Synergy 2) at 450nm and 540nm. 540nM was subtracted from the 450nm signal and a standard curve was generated to calculate sample concentrations. For Insulin, after washing the plate, 100 µL of TMB Substrate was added to each well and the plate incubated for 30 minutes at RT. 100 µL of Stop Solution was then added and the plate was immediately analyzed at 450nm to generate a standard curve and calculate sample concentration.

Luminex assay.
For protein isolation, Dulbecco's phosphate-buffered saline with protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN) was added to adipose tissue sample. Samples were then homogenized and centrifuged (2,000g x 5 minutes) to remove debris, and then the supernatant was centrifuged a second time (10,000g x 10 minutes). Supernatant was collected for quantitative protein analysis using Luminex multiple antigen magnetic bead assay (Luminex Corporation, Austin, TX) for IL-6, MCP-1, TNF-α, IL-8, Leptin and Adiponectin.

Statistical analysis.
Results are presented as Mean ± standard error of the mean (SEM).

For the gut microbiome analyses, raw sequences were processed using the Quantitative Insights Into Microbial Ecology (QIIME) software package and OTUs were picked at 97% similarity of the 16S rRNA gene.[27] A mean sequencing depth of 162,514 ± 24,267 (SEM) reads per sample was obtained. QIIME software packages and custom Python scripts were used to measure and analyze α and β diversity [27] to ensure that all samples contributed equally, sequences were subsampled to a common depth of 100,000 reads prior to β-diversity analyses. Due to limited sample size, we lacked power to employ robust statistical techniques. Nevertheless, to gauge shifts in the gut microbial community with treatment, we performed linear discriminant analysis effect size (LEfSe) analysis [28] to identify bacterial biomarkers that distinguished samples collected during the dietary intervention versus either before or after the dietary intervention. For LEfSe-identified microbial biomarkers, we performed a within-subjects comparison of relative abundance at baseline versus intervention period via paired t-test.

3. Results.

The study outline and design are portrayed in **Figure 1**. Out of a total 51 screened patients, 33 fulfilled enrollment criteria and 9 patients consented to the study. Of 9 consented patients, 3 patients withdrew due to scheduling problems related to the inpatient research unit setting, 1 patient was excluded due to newly discovered malnutrition (low pre-albumin). **Table 1** summarizes the various reasons for non-consent and rejection after initial screening, **Table 2** the patient characteristics after randomization. Of 5 patients enrolled, 4 where randomized in the PCR group and 1 in the AL group. The patient in the AL group was withdrawn at day 1 of the diet due to newly discovered substance abuse despite having undergone thorough pre-randomization screening. This medical issue was also not known to the attending surgeon who saw the patient in clinic, nor was it uncovered by the hospital's standard pre-operative evaluation process which involved a detailed history by advanced nurse practitioners. All 4 patients from the PCR group completed the diet and reported no noticeable side effects, and there were no compliance issues nor adverse events with the PCR diet. Post-operative duplex ultrasounds revealed no recurrent stenosis (all index operative carotids <50%) in all four patients at post-op day 30.

Clinical parameters (blood pressure, heart rate, temperature) and general lab (chemical, hematology, coagulation) performed at the various time-points during the course of the study all remained well within the expected range for that specific time-point (pre- versus post-surgery, data not shown). All PCR patients lost weight during the course of the diet, but remained within 5% of their starting weight (**Figure S1**).

ELISA assay showed that insulin levels remained within normal range[29] during the course of the study in all four patients (**Fig S2A**). Fibroblast Growth Factor 21 (FGF-21) is a hormone upregulated during fasting[30] and pivotal in the metabolic response in dietary restriction mouse models.[31] In our study population, due to lacking of sample size and randomized controls, no significant increase in circulating FGF-21 was could be detected after 3 days of PCR (**Fig S2B**). Luminex assays of serum at the various time-points and on subcutaneous and perivascular adipose tissue samples prior to the CEA procedure showed no abnormal cytokine or adipokine levels (**Figure S2C-I**).

In our microbiome study, LEfSe analysis revealed differential relative abundance of a single microbial taxon, *Bilophila wadsworthia,*[18 32, 33] between samples collected during the dietary intervention versus either before or after the dietary intervention (LDA > 2, **Fig. S3A**). Comparing baseline to post-treatment samples for the 3 of 4 patients that submitted a baseline sample, paired t-tests also indicated a trend toward increased relative abundance of *Bilophila wadsworthia* with treatment ($p = 0.08$). Notably, this increase in *Bilophila wadsworthia* with treatment was evident in all 4 patients (**Fig. S3B**).

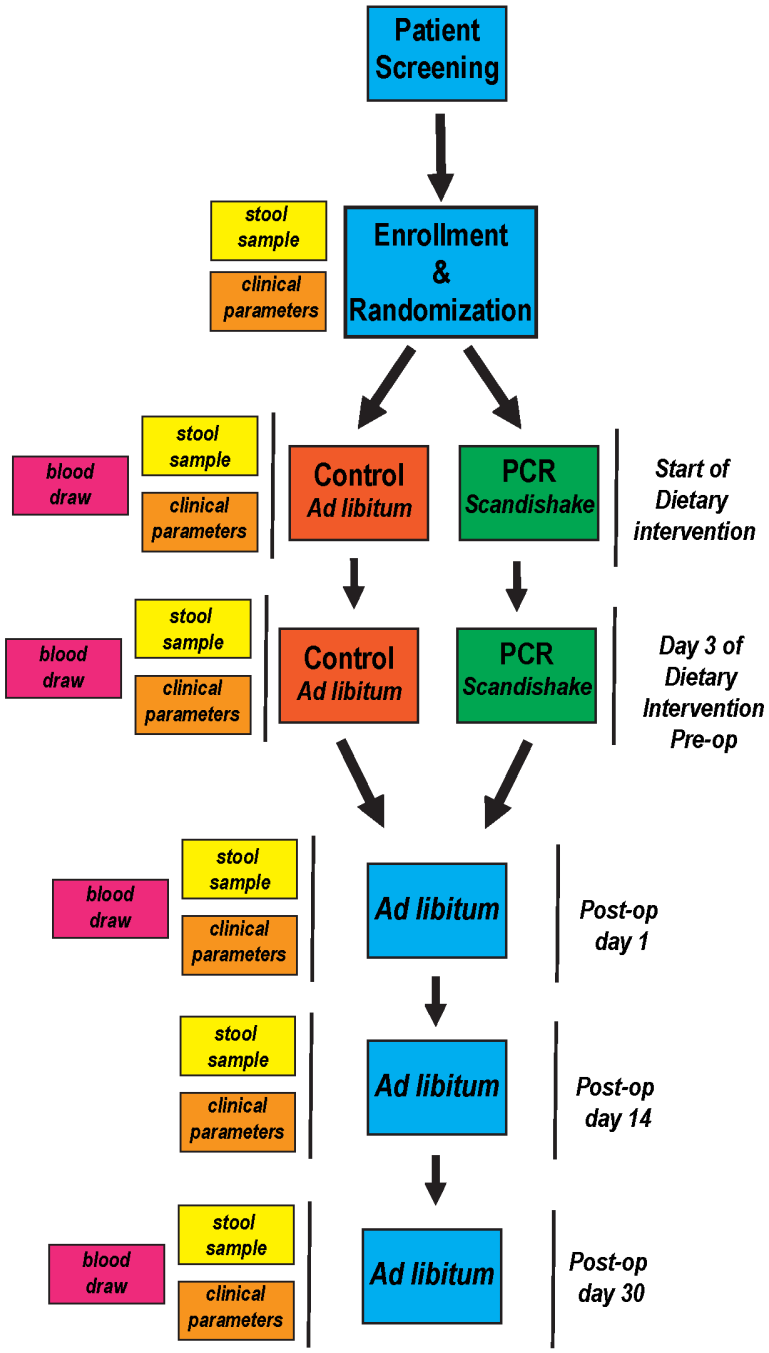


Figure 1. Study design

Table 1. Summary of consent, non-consent and exclusion after initial screening.

Initial Screening and Consent	
Total screened patients	**51**
Total consented	17.6% (n=9)
No available research unit	5.9% (n=3)
Malnutrition (low albumin)	2% (n=1)
Total randomized	9.8% (n=5)
Total non-consent	47% (n=24)
Burden of 3-day hospital stay	33.3% (n=17)
Lack of interest in research	13.7% (n=7)
Total rejected after initial screening	35.2% (n=18)
Active cancer	5.9% (n=3)
Deemed medically unfit	29.4% (n=15)

Table 2. Summary of patient characteristics. Patient 5 was discharged from the study due to the exclusion criteria of substance abuse.

Patient Characteristics	
Patient 1	***PCR***
General	69, female
Carotid disease	Asymptomatic right carotid stenosis
Co-morbidities / Medical history	Hypertension, gastric bypass, OSA, prediabetic
Patient 2	***PCR***
General	74, female
Carotid disease	Asymptomatic right carotid stenosis
Co-morbidities / Medical history	Lower extremity stent, PCI with DES, GERD, COPD, hypertension, hypercholesteremia, active smoker
Patient 3	PCR
General	67, female
Carotid disease	Symptomatic right carotid stenosis
Co-morbidities / Medical history	Recent embolic stroke, myocardial infarct, CABG, hypertension, diabetes type II, hypercholesteremia, chronic kidney disease
Patient 4	PCR
General	69, male
Carotid disease	Asymptomatic left carotid disease
Co-morbidities / Medical history	Myocardial infarct with DES, hypercholesteremia

4. Discussion.

It is well known that long-term reduction of calorie intake exerts the ability to extend life-span and improve health in a wide range of animal models.[34, 35] Such long-term dietary interventions are also effective in pre-clinical models of ischemia-reperfusion injury to the heart and brain.[36-38] In terms of feasibility and compliance, however, long-term dietary interventions appear to be less suited for implementation in everyday clinical practice including in the setting of elective vascular surgery.

Short-term preoperative dietary interventions however, i.e. ranging from one-week to as short as three days duration, display similar benefits in preclinical surgical models.[2-5, 8, 16, 39-41] Therefore holding greater promise as an intervention to improve vascular conduit durability, lower peri-procedural complications rates and improve overall metabolic health in vascular surgery patients scheduled for elective surgery,[39] ultimately diminishing the need for reintervention.[40] For example, a 3 to 6-day mild caloric restriction in patients with type 2 diabetes and severe insulin resistance was able to significantly reduce the insulin dose needed for glucose management.[42] Medicine (Baltimore

Additional studies have shown the feasibility of short-term pre-operative protein and/or calorie restriction in such diverse surgical patients populations as live kidney donors scheduled for transplant surgery,[21, 23] laparoscopic gastric bypass surgery,[22] elective cardiac surgery involving a cardiopulmonary bypass[24] and patients scheduled for liver resection.[43] It must be acknowledged that such a strategy to reduce morbidity and mortality would not be relevant in the emergency surgery setting, though understanding the biologic mechanisms of dietary interventions might suggest other approaches that could be used in emergency cases.

In this initial vascular surgery patient exploratory study, we selected elective CEA patients since the overall surgical stress is less than other vascular operations (open aortic surgery, lower extremity bypass). It is acknowledged that complication rates are relatively low for CEA procedures, but this initial step provided insights into the vascular patient population and the research infrastructure needed to do a peri-procedural dietary restriction trial. CEA in our institution is also a very standardized operation. We also opted for an in-patient dietary research setting to be able to closely and adequately monitor patient compliance, comfort and safety. Certainly, eventual economical translation will necessitate a less expensive venue, likely outpatient in the patient's home. And

while none of the included patients displayed or reported any negative side-effects on health and general well-being as a result of the instituted dietary intervention, we found that vascular surgery patient consent to the overall protocol was very low. The 3-day in-hospital stay, followed by medical fitness, constituted the main body of arguments for non-consent. For diet, we chose Scandishake as reported previously by the Rotterdam group.[23]

Our study was markedly limited by the difficulties of recruiting patients for an inhouse study, and therefore statistically hampered in being able to assess the effects of the intervention on endpoints of general and metabolic health such as circulating levels of insulin and FGF-21. Furthermore, measurement of circulating and perivascular adipokine production was intended as a secondary endpoint of dietary preconditioning efficacy, but remain uninterpretable due to the lack randomized control samples. Certainly, the late stage goals of trials in this area should focus on clinically meaningful per-operative and long-term events such as wound complications, myocardial infarction, stroke, revascularization durability, and survival.

In the microbiome arm of our study, however, the singular emergence of *Bilophila wadsworthia* is intriguing given that this species is among a small group of sulfite-reducing, hydrogen sulfide producing bacteriia.[33] An interesting correlation given our recent discovery of increased endogenous H_2S production in the benefits derived from dietary preconditioning in preclinical surgical models.[11, 16] Nevertheless, our confidence in this result is necessarily limited by the small sample size of 4 patients, none of whom contributed samples at all time points, and all of whom were on the treatment. Without control samples, we cannot be certain that the increase in *Bilophila wadsworthia* arose as a result of the diet treatment, as opposed to other environmental factors or interventions associated with hospital admission. However, our data do generally support host-microbial interactions in hydrogen sulfide metabolism occurring in parallel with or as a result of treatment.

In conclusion, since no concerning effects on health or compliance with the diet were observed, for future vascular surgery PCR clinical studies we will move towards an out-patient setting for patients undergoing a wider variety of elective vascular surgery interventions, including endarterectomies, aortic reconstructions, extremity bypasses and even hybrid open-endovascular approaches. The multi-disciplinary research processes to examine potential pleotropic mechanisms of PCR protection functioned well in this pilot study

setting though investigational success with the additional complexities of the outpatient setting remains to be determined. Continued steps to translate short-term pre-procedural nutritional interventions stand as a pressing medical research mandate to mitigate the human and economic toll of vascular surgical procedures.

Final changes in the percentage of 16S rRNA gene sequencing reads mapped to *Bilophila wadsworthia*. All four patients are shown as individual graphs since each displayed different relative abundance of *Bilophila wadsworthia* at each time point and because samples were not collected at all time points. Paired T-test of relative abundance of *Bilophilia wadsworthia* pre-diet versus after-diet samples was performed. (Pre-diet: 0.001±0.00077 versus After-diet: 0.0182±0.007; P=0.08). Data is represented as Mean±SEM.

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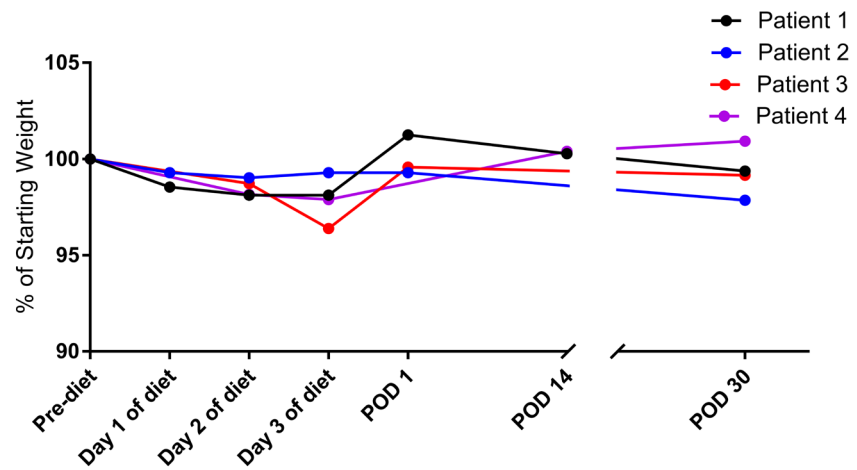


Figure S1. Weight loss during the study as percentage of starting weight. All patients remained within 5% of their starting weight.

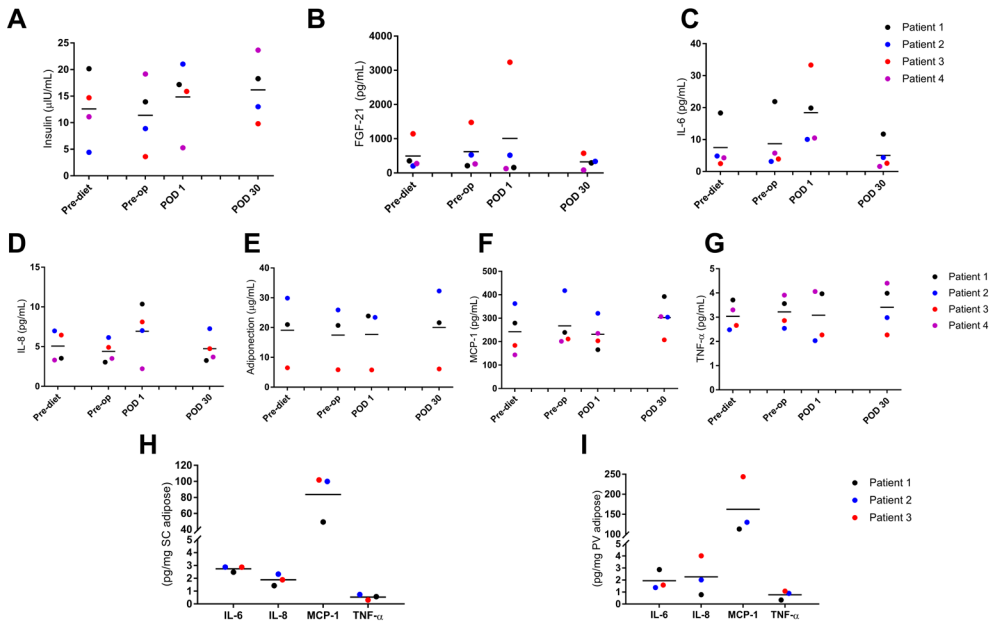


Figure S2. A: Insulin levels in μU/mL in all 4 patients during the course of the study, as measured by ELISA. **B:** Circulating FGF-21 levels in pg/mL during the course of the study, as measured by ELISA. **D-G**: Circulating levels of MCP-1, TNFα, IL-8 and adiponectin during the course of the study. **H**: IL-6, IL-8, MCP-1 and TNF-α levels in the subcutaneous adipose tissue, sampled during the carotid endarterectomy procedure. **I**: IL-6, IL-8, MCP-1 and TNF-α in the perivascular adipose tissue surrounding the carotid artery, sampled during the carotid endarterectomy procedure.

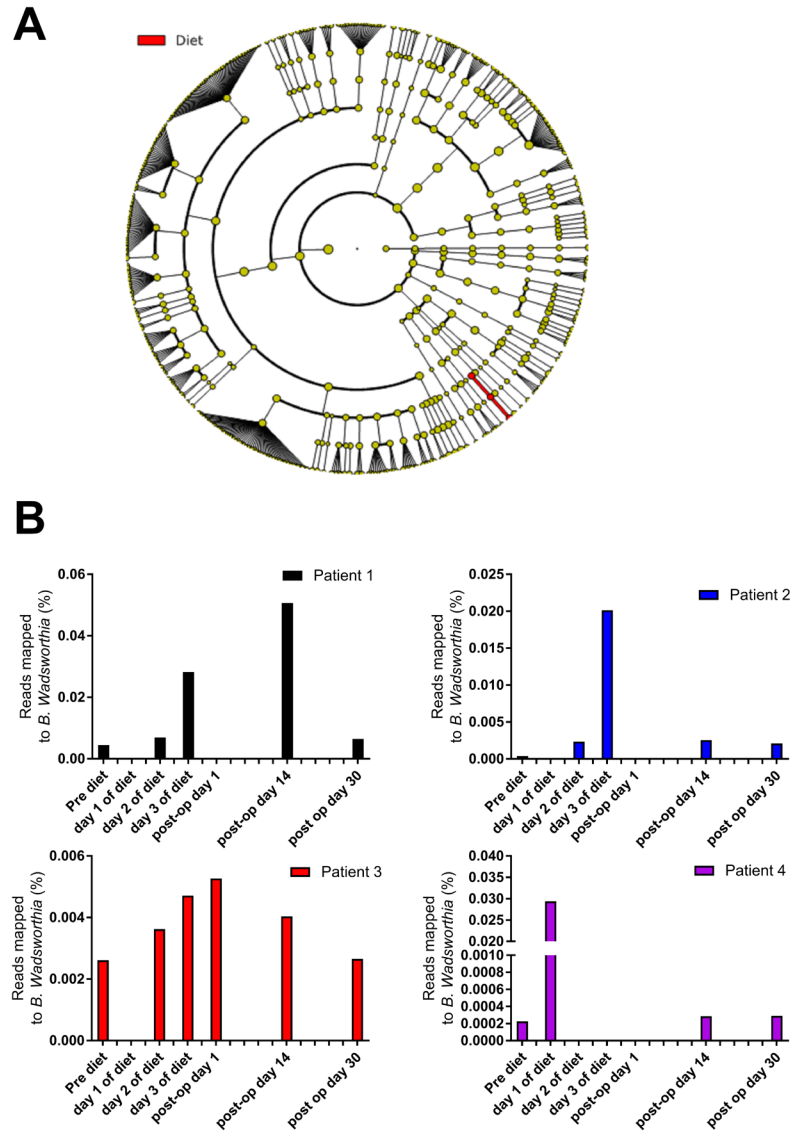


Figure S3. A: Differentially abundant bacterial taxa between the samples collected during the dietary intervention (n = 9) and the samples collected either before or after the dietary intervention (n = 11). Cladogram generated by LEfSe indicating differential relative abundance of *Bilophila wadsworthia* as the sole taxon for which LDA > 2. **B:** Patient-specific longitud

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Chapter 7.

Short-term Pre-operative Protein Caloric Restriction in Elective Vascular Surgery Patients: A Randomized Clinical Trial

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Abstract.

Background
Vascular surgery operations are hampered by high failure rates and frequent occurrence of peri-operative cardiovascular complications. In pre-clinical studies, pre-operative restriction of proteins and/or calories (PCR) has been shown to limit ischemia-reperfusion damage, slow intimal hyperplasia, and improve metabolic fitness. However, whether these dietary regimens are feasible and safe in the vascular surgery patient population remains unknown.

Methods
We performed a randomized controlled trial in patients scheduled for any elective open vascular procedure. Participants were randomized in a 3:2 ratio to either four days of outpatient pre-operative PCR (30% calorie, 70% protein restriction) or their regular *ad libitum* diet. Blood was drawn at baseline, pre-operative, and post-operative day 1 time-points. A leukocyte subset flow cytometry panel was performed at these time-points. Subcutaneous/perivascular adipose tissue was sampled and analyzed. Follow-up was one-year post-op.

Results
19 patients were enrolled, of whom 11 completed the study. No diet-related reasons for non-completion were reported, and there was no intervention group crossover. The PCR diet induced weight loss and BMI decrease, without malnutrition. Insulin sensitivity was improved after 4 days of PCR (p=0.05). Between diet groups there were similar rates of re-intervention, wound infection and cardiovascular complications. Leukocyte populations were maintained after four days of PCR.

Conclusions
Preoperative PCR is safe and feasible in elective vascular surgery patients.

Key words: Dietary restriction, vascular surgery, metabolic fitness

1. Introduction.

Vascular surgery patients suffer from peri-operative complications, such as myocardial infarction, ischemic stroke [1], and infections [2] at rates higher than those in many other surgical populations. Additionally, numerous procedure-specific challenges exist in vascular surgery. For example, in patients undergoing revascularization for peripheral artery disease (PAD), loss of graft patency is a constant threat. Over 200 million individuals worldwide suffer from PAD [3]. In the past decade, the number of lower extremity interventions for PAD has nearly doubled [4], and revascularization procedures are a high-volume mainstay in treating arterial occlusive disease [5]. Unfortunately, interventions for PAD are associated with high failure-rates [6, 7], resulting in frequent re-admission [8], and often requiring re-interventions. [6, 7] Such complications can lead to enormous patient suffering, and even death, ultimately resulting in an immense economic and social burden [9]. Therefore, new strategies to improve peri- and post-op complication rates following vascular surgery are urgently needed.

Some progress in treating vascular surgery patients has been made by implementing Enhanced Recovery After Surgery (ERAS) protocols [10], which have been demonstrated to reduce post-operative inpatient length of stay [11]. Key parts of these protocols focus on optimizing nutrition in the peri- and postoperative time-period, together with early patient mobilization, but guidelines make few recommendations concerning optimal preoperative nutrition. Interestingly, precise requirements for “optimal” preoperative nutrition are not globally agreed upon, and recent evidence has emerged that a more extensive dietary preconditioning strategy, such as protein restriction, during the days-weeks leading up to the procedure could potentially profoundly impact post-operative outcomes [12]. This concept of dietary restriction (DR), i.e. restriction of calories, proteins, specific amino-acids or a combination of the aforementioned – but without malnutrition, is best known for its ability to increase health and lifespan in various species when applied long-term (months-years)[13].

Various forms of DR, including caloric and protein restriction, can modulate the physiologic stress response to surgical injury which likely underlies peri-operative complications and contributes to the limited lifespan of some vascular reconstructions. Short-term DR, with durations spanning 3 days to 4 weeks, has been demonstrated to enhance recovery of renal function [14-16] and mitigate hepatic damage [14, 16-18] after surgical ischemia-reperfusion injury.

Surgical stress signaling in soft tissues, such as adipose [19], is also attenuated by DR [20]. Importantly, short-term DR does not appear to impair wound healing [21]. In vascular surgery models, DR attenuates arterial intimal hyperplasia [15], improves vein graft remodeling after rodent bypass surgery [22], and stimulates angiogenesis after femoral artery ligation [23].Interestingly, combining two forms of DR (i.e. protein and caloric restriction, [PCR]) appears more efficacious in yielding protection after surgical stress than unilateral restriction of calories or proteins [24]. Mechanistically, one of the mechanisms through which DR confers its benefits in various surgical models can be explained by upregulation of endogenous hydrogen sulfide (H_2S) [16], a gaseous vasodilator [25] with broad anti-inflammatory [26], antioxidant [27] and anti-atherosclerotic [28, 29] properties.

In a prior pilot study, patients undergoing elective carotid endarterectomy [30] underwent a preoperative PCR intervention while being closely monitored as inpatients for three days prior to scheduled surgery, to assess the safety of this specific diet in this fragile populatio. All patients completed the study without experiencing any adverse events or dietary compliance issues. In the current study, we focused on evaluating the feasibility of a pre-operative PCR diet in the vascular surgery patient population. To test this, we optimized the study design to be more translationally relevant by performing it in an outpatient population and including all open vascular surgery operations. The current study assesses the feasibility of and compliance with a short-term PCR diet in a cross-section of vascular surgery patients. Additionally, we investigated the effects of a preoperative PCR diet on clinical parameters and metabolic health. Furthermore, we interrogated the innate and adaptive immune response to the diet and the surgical intervention. Specificall, evaluating the effects of the diet on both the response to surgical stress and endogenous H_2S modulation in these immune cell subsets.

2. Material and Methods.

2.1 Trial Design and Setting.

This randomized controlled outpatient clinical trial was approved by the Partners Human Research Committee institutional review board, and registered with ClinicalTrials.gov (Identifier: NCT04013412), to enroll subjects at one academic tertiary medical center (Brigham and Women's Hospital, Boston MA USA). Study subjects were recruited from a cohort of patients who were scheduled to undergo an elective open vascular surgery procedure (defined below) at a single institution. After initial screening and patients were deemed eligible, written informed consent was obtained by a physician-investigator from subjects before enrollment. Patients were then randomized at their baseline visit (scheduled between 30- and 5-days pre-surgery) to either the *ad libitum* (AL) group or a 4-day pre-operative protein-caloric restriction (PCR) diet to be consumed in an out-patient setting. Patients continued their assigned diets for four days leading up to the scheduled surgery until midnight at the day of surgery, when both cohorts were instructed NPO (nil per os) except selected medications. On the day of surgery, patients underwent typical same day admit processes, and received postoperative care per standard clinical practice. Immediately post-op, all patients were advanced rapidly to an *ad libitum* diet as tolerated, and patients were followed prospectively until post-op day 30 (Figure 1, study design). This randomized controlled clinical trial took place from May 2019 until February 2020, when enrollment was terminated due to the global COVID-19 pandemic.

2.2 Inclusion and Exclusion Criteria.

Inclusion criteria.

Patients eligible for inclusion included all patients greater than 18 years old who presented for one of the following elective procedures: carotid artery endarterectomy, aortic/iliac aneurysm repair (open or endovascular only if groin cut down planned), open lower extremity arterial procedures (bypasses, aneurysm repair, arterial and bypass graft reconstructions), major amputations of the lower extremity (below knee and above knee amputations), and open hemodialysis access procedures.

Exclusion criteria.

Patients were excluded from our study for intolerance or allergy to any of the ingredients in the PCR diet, active infection, pregnancy, malnutrition (serum albumin < 3g/dL), uncontrolled diabetes (HgbA1c > 12%), substance dependency that could interfere with protocol adherence and assent as determined by the principal investigator, active non-cutaneous cancer under treatment with chemotherapeutics or radiation, emergency surgery, active participation in any other interventional or randomized study, and participation in the current study within the past 30 days.

2.3 Randomization and Intervention.

After written informed consent was obtained, patients were randomized in a 3:2 ratio by parallel design. We opted for a 3:2 randomization design to rigorously study diet compliance and safety in the PCR group, while maintaining sufficient numbers of AL patients for controls. When randomized to the PCR group (Scandishake [any of 4 flavors: vanilla, strawberry, banana cream or caramel] mixed with almond milk) patients received their PCR diet for the next four days, which was calculated individually (see "Dietary Compliance") as a total daily volume to achieve 30% caloric restriction and 70% protein restriction based on body weight and activity level. For the macronutrient ratio of Scandishake in Kcal and percentage, see Table 1.

When allocated to the AL group, patients could continue their *ad libitum* diet.

Table 1. Scandishake macronutrient ratio in Kcal and %

Macronutrients	Kcal	%
Protein	19.64	4
Carbohydrates	255.32	42
Fat	216.04	44
Total	491	100

2.4 Blinding.

All non-essential clinical staff were blinded to the study arm, including basic science study staff, to ensure unbiased interpretation of clinically obtained data and specimens. All experiments and assays on blood and tissue samples were performed blinded, and study staff was unblinded after trial and specimen analysis was completed.

2.5 Dietary compliance.

Patients enrolled in the PCR group received 16 portions of the PCR Scandishake diet at their baseline visit to accommodate for a four-day outpatient PCR diet. For macronutrient ratio of the Scandishake diet, see table 1. To calculate the volume of PCR diet needed in order to achieve a 30% caloric- and 70% protein restriction, qualified dieticians employed the Mifflin St. Jeor equation [31] based on gender, age, height, weight and activity factor and combined this with the patients last meal recall. To monitor diet compliance in the PCR group and food intake in the AL group, a MealLogger app was utilized for study subjects to self-record their intake. The PCR group was encouraged to monitor anytime they deviated from the PCR diet.

2.6 Clinical parameters.

At the baseline visit, immediately preop, post-op day 1 (POD1), and POD30, study participants' height, weight (utilizing the same scale for all patients/timepoints), temperature, blood pressure and heart rate was recorded. Clinically important perioperative outcomes including mortality (all cause and cardiovascular), stroke, myocardial infarction, coronary revascularization, thrombotic complications, and reinterventions related to the index case were determined through review of the electronic health record.

2.7 Blood Draws.

Fasting blood draws were performed at the baseline clinic visits, immediately preop on POD0, and on POD1, and collected in designated tubes. At each time-point, 5.0 mL of whole blood was collected in an ethylenediaminetetraacetic acid (EDTA) covered tube for a complete blood count with differential, and 10mL of whole blood was collected in a red top tube (RTT) for a basic metabolic panel combined with cortisol, insulin growth factor-1 (IGF-1) and c-reactive protein (CRP). Baseline and preop testing also included pre-albumin. These tests were all performed by technicians at the Center for Clinical Investigation at Brigham and Women's Hospital and tested by the laboratory Corporation of American Holdings (LabCorp). A second RTT with 8.0 mL of whole blood was collected. After incubation for 30 minutes on wet ice, the tube was centrifuged (Thermo Forma 5681 3L GP) at 2500 x *g* for 15' at 4°C. Supernatant was then collected in several 1.5mL Eppendorf tubes and stored as serum at -80°C until further analysis. At each time-point, a second EDTA tube with 8.0 mL of whole blood was also collected at room temperature (RT) and immediately processed for flow cytometry analysis as described below.

2.8 Adipose Tissue Biopsy.

At the start of the surgical procedure, immediately following the first incision, a subcutaneous adipose tissue sample of approximately 1cm^3 was collected in a 1.5mL Eppendorf tube and flash frozen in liquid nitrogen. Next, when the surgical field around the target vessel was explored, approximately 1cm^3 of arteriovenous perivascular adipose tissue was collected in a 2.0mL Cryotube and flash frozen in liquid nitrogen. Both samples were then stored at -80°C until further processing for Luminex analysis.

2.9 Flow Cytometry Panel Creation & Validation.

To characterize lymphocyte and monocyte subsets in the blood of study participants before and after diet and surgery, a comprehensive antibody panel, processing and gating strategy was developed based on a previously published panel [32]. First, antibodies (listed below) were titrated on healthy donor human peripheral blood mononuclear cells (PBMC) to determine the optimal staining index, resulting in the following antibody cocktail described in Table 1.

Table 2. Antibody cocktail for the study.

Antibody (mouse α human)	Important marker for	Fluorophore	Clone	Manufacturer	Catalog number	Final volume (in 100µL)
CD3	T-cells	AF-700	UCHT1	Invitrogen	56-0038-42	5 µL
CD4	CD4 T-cells	PercP-Cy5.5	RPA-T4	BD Biosciences	560650	1.25 µL
CD8a	CD8 T-cells	BV785	RPA-T8	Biolegend	301046	5 µL
CD14	Monocytes	PE-Cy7	M5E2	BD Biosciences	560919	5 µL
CD16	Monocytes	PE-CF594	3G8	Biolegend	302054	2.5 µL
CD19	B-cells	BV650	HIB19	Biolegend	302238	10 µL
CD25	Treg-cells	APC	M-A251	BD Biosciences	590987	20 µL
CD38	T-cell/B-cell	AF 488	HIT2	Biolegend	303512	2.5 µL
CD56	B-cells	BV605	HCD56	Biolegend	318334	5 µL
CD127	Treg-cells	BV711	A019D5	Biolegend	351328	5 µL
CD183 (CXCR3)	Th1 cells	PE	11A9	BD Biosciences	743356	10 µL
CD196 (CCR6)	Th1, Th2, Th17	BUV395	1C6/ CXCR3	BD Biosciences	560928	1.25 µL
HLA-DR	Dendritic cells	PE	LN3	Invitrogen	47-9956-42	0.625 µL

This panel was combined with a fluorescent probe that binds free H_2S[33]: P3 (EMD Millipore, cat#534329, 378/524 nm [Ex/Em]), and a Zombie Violet Fixable Viability live/dead dye to exclude dead cells (Biolegend, cat#423113, 400/423 nm [Ex/Em]).

For single compensation control of non-abundant epitopes (i.e., CD25, CD38, CD56, CD127, CD183, CD196), the following CD4 antibodies were used as described in Table 2.

Table 3. CD4 antibodies

Antibody (mouse α human)	Fluorophore	Clone	Manufacturer	Catalog number	Final volume (in 100µL)
CD4	APC	RPA-T4	BD Biosciences	561840	20 µL
CD4	BUV395	RPA-T4	BD Biosciences	564724	1.25 µL
CD4	PE	RPA-T4	BD Biosciences	561843	20 µL
CD4	BV605	RPA-T4	Biolegend	300556	2.5 µL
CD4	AF488	RPA-T4	Biolegend	300519	5 µL
CD4	BV711	RPA-T4	Biolegend	300558	2.5 µL

Fluorescence minus one (FMO) control were employed for the following epitopes: CD25, CD38, CD56, CD127, CD183, and CD196. An FMO was also run for P3. To account for inter-patient and inter-timepoint variability, healthy donor PBMCs were cryopreserved and thawed (see below) during patient blood processing to employ as single compensation FMO controls.

2.10 Isolation of Control Peripheral Blood Mononuclear Cells from Healthy Donors.

We isolated PBMCs from healthy donors and cryopreserved these cells to titrate and validate our antibody panel, as well as to use in our single control and FMO compensation assays. Approximately 250mL of healthy donor whole blood in EDTA tubes was acquired from Research Blood Products LLC Boston, delivered at RT. Upon delivery, whole blood was diluted 1:1 in 2% heat-inactivated fetal bovine serum (Hi-FBS) (Sigma, cat#F4135) in Hanks' Balanced Salt Solution (HBSS), without calcium or magnesium (Thermo Fisher, cat#14170112), i.e. washing buffer (WB). After careful mixing, the suspension was layered on top of an equal volume of Lymphoprep density gradient medium (Stem Cell, cat#07811) in a 50mL tube and centrifuged at 800 x *g* for 20′ at RT with brakes off. Afterwards, using a sterile dropper pipet the PBMC layer was collected in a new 50mL tube and washed with WB. PBMCs were next centrifuged at 400 x *g* for 10′ at RT with brakes on. The tube was decanted, flicked 3 times, and the PBMC pellet was resuspended in 50mL WB. The tube was again centrifuged at 400 x *g* for 10′ at RT, then resuspended in WB and counted before subsequent cryopreservation.

2.11 Peripheral Blood Mononuclear Cell Cryopreservation.

10% Dimethyl sulfoxide (DMSO, Sigma, cat#D2650-100ML) was mixed with 90% Heat-Inactivated Fetal Bovine Serum (Hi-FBS, Sigma, cat#F4135) to create cryopreservation solution (CPS) and cooled on wet ice, together with Nunc Cryotubes (Thermo Fisher, cat#377267), before start. Freshly isolated PBMCs were pelleted and resuspended in CPS, and then aliquoted into cryovials at a final concentration of $1x10^7$ PBMCs/mL. Cryovials were then immediately transferred to a Nalgene Mr. Frosty cryo-freezing container (Thermo Fisher, Cat#5100-0001) and stored at -80°C for 24 hours before transfer to a liquid nitrogen (LN_2) tank in vapor phase for longer term storage.

2.12 Peripheral Blood Mononuclear Cell Thawing.

PBMCs of healthy donors were thawed at regular intervals to function as single compensation and FMO controls for patient whole blood. Thawing media consisted of RPMI Complete (RPMI1640 with 10% Hi-FBS with 200 IU Penicillin, 200 µg/mL Streptomycin and 2 mM L-Glutamine) and was pre-warmed before start. One cryovial was taken from LN_2 storage and immediately transferred to a 37°C water tank and thawed with regular flicking of the tube. The cryovial was transferred to a biosafety hood when only a small bit of ice remained, after which 1mL of thawing media was added in a dropwise manner. The solution was then transferred to a 15mL tube and 10mL of thawing media was slowly added. The tube was then centrifuged at 400 x *g* for 10′ at RT. The pellet was resuspended in 15mL of thawing media and again centrifuged at 400 x *g* for 10′. The pellet was resuspended in 1mL of running buffer and thawed healthy donor PBMCs were then used for single compensation and FMO controls in flow cytometry analysis of the study participants' samples.

2.13 Processing of Patient Whole Blood for Flow Cytometry Analysis.

At the baseline, immediately preop, and POD1 time-points, 8.0mL of patient whole blood was collected in an EDTA tube via vein puncture and kept at RT. This tube was then immediately processed for flow cytometry analysis. Firstly, 2mL of whole blood was transferred to a 50mL tube with 25mL of Ammonium-Chloride-Potassium (ACK) buffer pre-warmed to 37°C to lyse erythrocytes. The tube was incubated for 12′ with tube inversion every 2′. ACK buffer was produced by mixing 150mM NH_4Cl (Sigma, cat# 254134), 10mM $KHCO_3$ (Sigma, cat# 237205) and 0.1mM Na_2EDTA (Sigma, cat# 324503) with 850mL of H_2O, then pH was adjusted to 7.2-7.4 before achieving a final volume of 1000mL. After 12′ incubation with ACK lysing buffer, the tube was filled until 50mL with pre-warmed running buffer (0.05% bovine serum albumin in diH_2O) and spun down at 400 x *g* for 5′ at RT. The supernatant was then decanted, and the tube was flicked three times before resuspending the white blood cell (WBC) pellet in 50mL running buffer. The tube was again spun down at 400 x *g* for 5′ at RT before the supernatant was decanted and the pellet resuspended in 0.5mL running buffer. To acquire a WBC-count, the sample was diluted 1:20 and loaded onto a hemocytometer. The four outer squares were counted and averaged. WBCs were counted twice, and the final count was averaged. $1x10^6$ WBCs were then loaded per well on a 96-well round-bottom plate (Thermo Fisher, cat#475434) for further downstream flow cytometry processing.

2.14 Staining Procedure of WBC & PBMCs for Flow Cytometry.

After patient blood was acquired, lysed, and resuspended as $2x10^6$ WBC/well in phosphate buffered saline (PBS) in a 96-well round bottom plate, thawed PBMCs were added in the same plate with PBS ($0.15x10^6$ PMBCs/well). Each plate was centrifuged at 400 x *g* for 5′ at RT, decanted, and pellets were resuspended in Zombie live/dead dye (1:100, in PBS). Plates were incubated on wet ice in the dark for 15′, then washed with running buffer and again centrifuged. Pellets were then resuspended in 50µL 4% rat serum (in running buffer) and incubated for 10′ on wet ice in the dark. Antibody cocktails (all stained and FMO's) were prepared, 10µL Brilliant Violet staining buffer was added (BD Biosciences, cat563794) together with running buffer resulting in a final volume of 99µL per cocktail. After incubation in rat serum, plates were centrifuged, decanted, and resuspended in antibody cocktail. Before incubation, P3 was added in a final concentration of 0.3µM per well. Plates were then incubated for 20′ on ice in the dark. Wells were then washed with running buffer, centrifuged and resuspended in 2% paraformaldehyde (diluted from 32% solution, Acros Organics, cat#AC416785000) in running buffer and incubated for 20′ at RT in the dark. Plates were then transferred to 4°C and run on the flow cytometer same day or next day.

2.15 Flow Cytometer Data Acquirement and Gating Strategy.

Immunolabeled white blood cells were analyzed by flow cytometry using a BD LSR II Special Order Research Product (SORP) flow cytometer with BD High Throughput Sampler (HTS), running DiVa software version 8.01, and equipped with excitation laser lines at 488 nm (20mW), 405 nm (50mW), 594 nm (200mW) and 355 nm (20mW). The 594 nm line was operated at 125 mW.

Data analysis was performed in FlowJo software (BD Biosciences, Ashland OR), version 10. Cells were delineated using the following gating strategy to identify live single cells:

FSC-A versus SSC-A bivariate contour plots were used to initially gate on lymphocyte, monocyte and granulocyte clusters. These populations were then visualized in an SSC-A versus Zombie Aqua fluorescence plot, with live cells gated as Zombie "negative". The live cells were next shown in FSC-A versus FSC-H plots, and single cells were selected.

In the experiments involving patient samples, 10^6 events, gated on live singlet populations, were collected for each stained experimental sample. 10^5 events were collected from above populations for each FMO, and 10,000 events were collected for samples stained with each individual antibody or dye for the purpose of compensation.

2.16 Luminex assay.

For protein isolation, Dulbecco's phosphate-buffered saline with protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN) was added to each adipose tissue sample. Samples were then homogenized and centrifuged (2,000g x 5') to remove debris, and then the supernatant was centrifuged a second time (10,000g x 10'). Supernatant was collected for quantitative protein analysis using Luminex multiple antigen magnetic bead assay (Luminex Corporation, Austin, TX) according to the manufacturer's instructions. For analysis of serum samples, the following panel was used: neuronal growth factor, interleukin-6 (IL-6), insulin, leptin, interleukin-8 (IL-8), monocyte-chemoattractant protein-1 (MCP-1), tissue necrosis factor-alpha (TNF-α), interleukin-1β (IL-1β), adiponectin, lipocalin, resistin, adiposin, plasminogen-activator inhibitor-1 (PAI-1). For analysis of adipose tissue samples, the following panel was used: NGF, IL-6, leptin, IL-8, hepatocyte growth factor (HGF), MCP-1, TNF-α, resistin, IL-1β, PAI-1.

2.17 Statistical analysis.

Based on the goals of the study, the patient enrollment was not powered to test a specific mechanistic hypothesis or efficacy, but rather to define infrastructure logistics, feasibility and the general safety of outpatient PCR in vascular surgery patients needing an open operation.

Data are expressed as mean ± standard deviation (Mean ± SD). Statistical testing was conducted with Student's T-tests and two-way ANOVA with Sidak's multiple comparisons for continuous variables, and Fisher's exact test for categorical variables. Kaplan-Meier survival functions were generated and univariable Cox regression performed for time-to-event outcomes. Statistical analyses were performed with Graphpad (8.12) and R (4.0.5, R Foundation for Statistical Computing, Vienna, Austria).

3. Results.

The study outline and design are depicted in **Figure 1**. From April 2019 until February 2020, 19 patients scheduled for elective vascular surgery consented to the study and were randomized. Out of 19 individuals, 12 were allocated to the PCR group and 7 patients to the AL group. **Table 1** compares patient characteristics of both groups. Out of 12 PCR patients, 8 completed the study, while out of 7 AL patients, 3 completed the study. **Table 2** summarizes the reasons for non-completion, which all occurred during the baseline stage of the study. Reasons for non-completion were either related to patient health (cardiac health, toxicology screening failure, emergency re-scheduling) or logistics (cancellation of surgery, missed baseline visit). Most importantly, none of the study participants reported issues with diet-compliance or tolerance, nor was there a diet-related withdrawal from the study.

Median follow-up was 504 days (IQR 385-715) using the reverse Kaplan-Meier technique, and follow-up at one year was 72.7%. No adverse events differed significantly between experimental groups in univariable Cox regression. Specifically, reintervention rates were similar between diet groups (HR 1.65, 95% CI 0.17 - 16.5, p = 0.67). In the PCR group, one patient suffered a myocardial infarction on POD3 after carotid endarterectomy, and one patient died due to a COVID infection one month after surgery. **Supplemental table 1** summarizes all AEs from date of index surgery.

Table 3 lists average energy and protein intake per kilogram per day in both the AL and PCR group at baseline and during the period the PCR group was subjected to the dietary intervention. The PCR group appeared to have lost weight at the preoperative visit (**Figure 2A**). The same trend was noted when comparing BMI at baseline with their pre-operative BMI (**Figure 2B**), in the PCR group. In terms of diet compliance, both the MealLogger data and weight

parameters are indicative of adherence to the PCR diet. To test whether our PCR diet would result in malnutrition, we measured baseline and pre-op levels of pre-albumin, a short half-life protein [34]. **Figure 2C** shows no difference in pre-albumin levels between groups and before/after the diet, suggesting patients continued adequate nutrition during the PCR intervention. In our study, we were not able to detect a difference in baseline and pre-op glucose levels (**Figure 2D**). However, we did see a trend towards increased insulin sensitivity as a result of the PCR diet at the pre-operative time-point as measured by insulin levels (**Figure 2E**).

We also investigated circulating and local cytokine and adipokine regulation pre- and post-surgery in response to the diet, as DR is known to reduce inflammation. We did not detect changes in any of the circulating cytokines (NGF, IL-6, IL-8, MCP-1, TNF-α or IL-1β; **Figure 3A**, IL-8 shown as an example) or adipokines (adiponectin, lipocalin, resistin, adiposin or leptin; **Figure 3B**, Leptin shown as an example) measured in serum at baseline, pre-operatively, and post-op. However, plasminogen activator inhibitor-1 (PAI-1) appeared to be increased in pre-operative PCR patients compared to baseline levels (**Figure 3C)**. Additionally, we investigated regulation of these same markers at the perioperative timepoint in both subcutaneous and perivascular (PVAT) adipose tissue. We detected no apparent change in levels of the aforementioned cytokines and adipokines (**Figure 3D, E**). Interestingly, PAI-1 was significantly downregulated in the PVAT of PCR patients, compared to patients who were AL fed (**Figure 3F**).

Table 1. Baseline Patient characteristics, post-randomization. Age-differences between groups was tested via student's T-test. Between-group differences of all other patient characteristics were tested via Fisher's exact test.

Baseline characteristics	AL (4)	PCR (12)	Statistical difference
Age in years (SD)	66.5 (11.3)	64.3 (12.8)	P=0.75
Gender Male (total)	2 (4)	8 (12)	
Female (total)	2 (4)	4 (12)	P=0.54
Smoking (total)	1 (4)	4 (12)	P=0.83
Diabetes (total)	2 (4)	4 (12)	P= 0.54
Hypertension (total)	4 (4)	9 (12)	P=0.51
Hypercholesterolemia (total)	1 (4)	4 (12)	P>0.99
History of Malignancy (total)	0 (4)	2 (12)	P>0.99
Transient ischemic attack / Stroke (total)	1 (4)	2 (12)	P>0.99
Cardiovascular disease	2 (4)	2 (12)	P=0.22
Peripheral vascular disease	2 (4)	7 (12)	P>0.99
Renal insufficiency	1 (4)	2 (12)	P=0.53

Table 2. Reasons for non-completion after initial consent and randomization. *Transcarotid artery revascularization (TCAR)*

Reasons for non-completion of the study	AL (7)	PCR (12)
Procedure changed to TCAR (outside of protocol at the time)	*1*	
Failed toxicity screening	1	
Patient failed to show for baseline, did not want to reschedule		1
	1	
Surgery cancelled		1
Failed pre-operative cardiac clearance	1	1
Surgery rescheduled emergently	1	
Patient opted out after baseline (did not want to deal with research, food diary, etc. before surgery)		1
Remaining study participants who completed the trial	3	8

Table 3. Energy and protein intake in AL and PCR groups.

	PCR	AL
Baseline energy intake	*23.04 Kcal/kg/day*	*11.97 Kcal/kg/day*
Intervention energy intake	*15.4 Kcal/kg/day*	*20.08 Kcal/kg/day*
Baseline protein intake	*1.06g/kg/day*	*0.34g/kg/day*
Intervention protein intake	*0.16g/kg/day*	*0.76g/kg/day*

We assayed circulating leukocyte subsets at baseline, preoperative, and post-operative timepoints in combination with a hydrogen sulfide (H_2S) probe that detects intracellular levels of H_2S.[33] **Supplemental figure S1** highlights the gating strategy employed to interrogate the different leukocyte subsets, based on a previously published strategy. [32] None of the interrogated cell populations (granulocytes, monocyte subtypes, T-cell subsets, NK cells, dendritic cells, and B-cells) showed any differences between diet groups and before/after surgery (**Supplemental figure S2**).

Lastly, we measured intracellular H_2S in each separate leukocyte subset. Although there was no detectable difference in H_2S levels between diet groups and time-points, we were able to make some observations. Intriguingly, cells considered part of the innate immune system consistently had higher levels of intra-cellular H_2S compared to cells of the adaptive immune system (**Fig. 4A**). Secondly, although classical pro-inflammatory monocytes possess high levels of H_2S, their non-classical anti-inflammatory counter parts have significantly lower levels of H_2S (**Fig 4A**), perhaps suggestive of a role for H_2S in mediating the inflammatory state of this cell type.

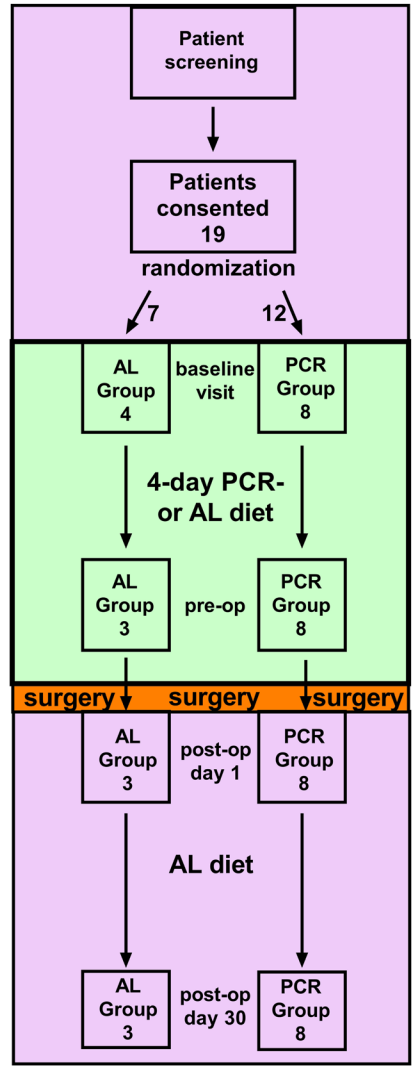


Figure 1. *Study design.* After consent, patients were randomized into *ad libitum (AL)* or protein calorie restriction (PCR) groups. Patient characteristics, clinical parameters, and blood were collected at the baseline visit, pre-operative visit, and at post-op day 1 (POD1). Perioperatively, we collected perivascular (PVAT) and subcutaneous (SQ) adipose tissue. Prospective follow-up was conducted until POD30.

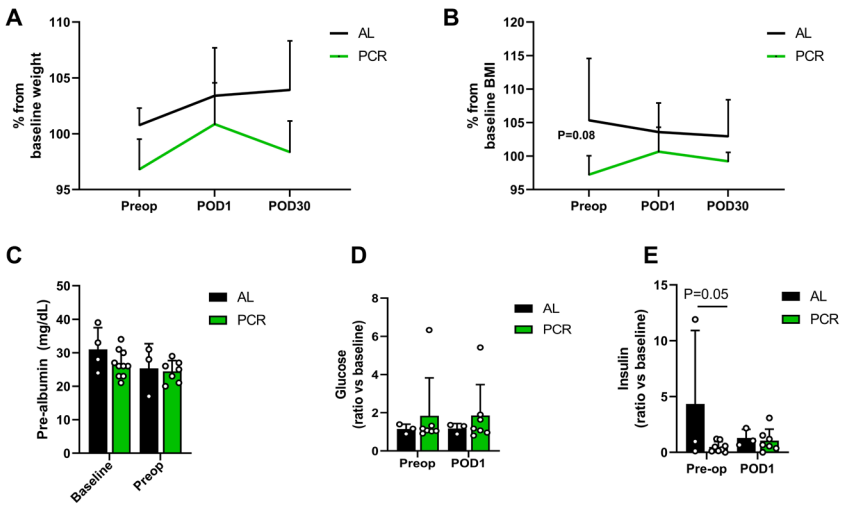


Figure 2. *The effects of a short-term PCR on patient weight and glucose homeostasis.* **A:** percent change in patient bodyweight at preop, POD1 and POD30 compared to weight at baseline visit. **B:** percent change in patient BMI at preop, POD1 and POD30 compared to BMI at baseline visit. **C:** pre-albumin levels (mg/dL) at baseline and preop in AL and PCR groups. **D:** patient blood glucose levels at preop and POD1, normalized to the respective patient's baseline blood glucose level. **E:** patient serum insulin levels at preop and POD1, normalized to the respective patient's baseline serum insulin level. Statistical testing was conducted via two-way ANOVA with Sidak's multiple comparisons test, n=3-10/group unless indicated otherwise.

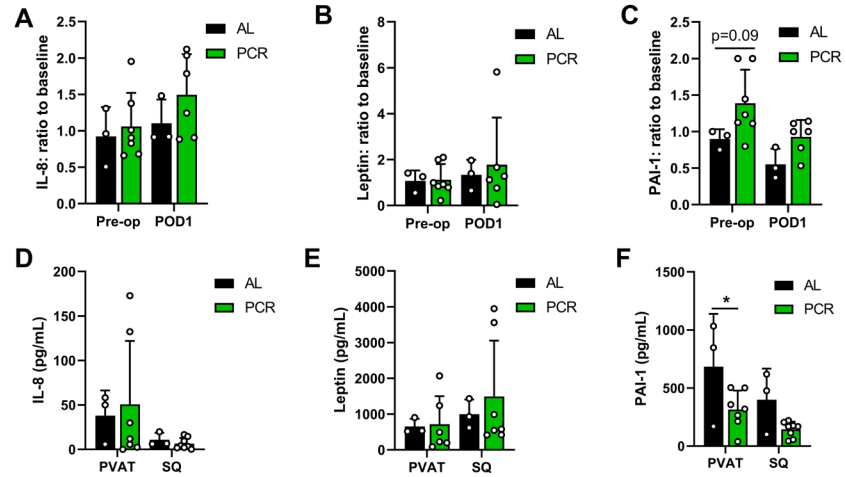


Figure 3. *The effects of short-term PCR on cytokines and adipokines, in circulation and in subcutaneous and perivascular adipose depots.* **A-C:** Cytokines and adipokines in serum of AL/PCR patients, baseline versus pre-op en POD1 ratio. **A:** Interleukin-8 (IL-8). **B:** Leptin**. C:** Plasminogen activator inhibitor-1 (PAI-1). **D-E**: Cytokines and adipokines in perivascular (PVAT) and subcutaneous (SQ) adipose tissue of AL/PCR patients. **D:** IL-8 in pg/mL. **E:** Leptin in pg/mL. **F:** PAI-1 in pg/mL. Statistical testing was conducted via two-way ANOVA with Sidak's multiple comparisons test, n=3-7/group unless indicated otherwise. * < 0.05

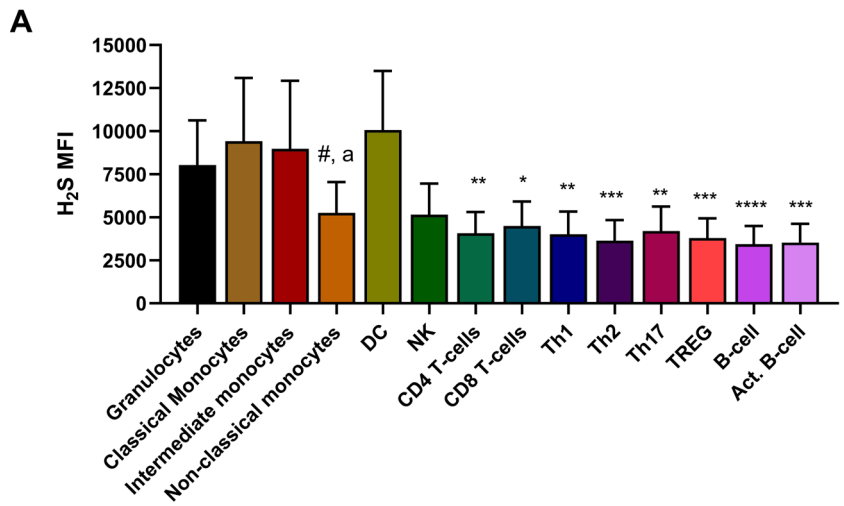


Figure 4. *Levels of endogenous intra-cellular H_2S differ between human innate and adaptive immune cells.* In the PCR group, all cell subtypes were compared at baseline for their intra-cellular levels of H_2S. Unless indicated otherwise, the differences between innate and adaptive cell types were highlighted via comparisons between granulocytes and each cell subset. Statistical testing was conducted via one-way ANOVA with Sidak's multiple comparisons test, n=6/group. * <0.05, ** <0.01, *** <0.001, **** <0.0001. # = classical monocytes vs non-classical monocytes, p = <0.01. a = intermediate monocytes vs non-classical monocytes, p <0.05

4. Discussion.

Here we present the results of a randomized controlled trial in outpatient pre-operative dietary restricted versus *ad libitum* fed patients scheduled for vascular surgery. This work expands on our previously published pilot study assessing the safety of such a short-term pre-operative PCR in inpatient vascular surgery patients [30]. Although the runtime of the trial and enrollment was shortened substantially due to the COVID-19 pandemic, we were still able to consent 19 individuals in 10 months. Ultimately, 11 patients completed the study, and we observed no diet-related reasons for withdrawal from the study once enrolled. The dietary intervention was well tolerated, with none of the study participants reported deviation from their PCR diet. The eight patients enrolled in the PCR arm achieved 29.4% calorie and 84.4% protein restriction on average, and these findings were supported by a decrease in weight and BMI in the PCR group compared to their baseline levels. There was no detectable difference in occurrence of adverse events between diet groups, although this trial was not designed to test efficacy but rather feasibility of this diet. Furthermore, nor were there any hypoglycemic or hyperglycemic incidents in the PCR group. Circulating leukocyte cell populations were maintained under PCR. Overall, these results indicate that short-term PCR in vascular surgery patients appears both feasible and safe.

Perioperative glucose regulation has been linked to improved outcomes and impaired wound healing in several studies [35, 36]. In a previous preclinical study, our group was able to link short-term pre-operative protein restriction with improved glucose homeostasis, both pre- and post-operatively [21]. In the current study, we were not able to detect any differences in pre- and post-op glucose levels. However, we did find lower pre-operative circulating insulin levels, suggestive of possible effect of PCR on glucose homeostasis/insulin signaling. A future, larger scale trial should be able to delineate whether short-term PCR improves glucose metabolism, and whether this can be linked to improved clinical outcomes and wound healing.

Intriguingly, PAI-1 levels were lower in the PVAT of patients after four days of PCR. Several studies have implicated adipocytes as the main producers of PAI-1, and production of PAI-1 in adipocytes is triggered by hyperglycemia and increased insulin resistance.[37] Indeed, PAI-1 is elevated in patients with diabetes type 2 compared to lean control subjects [38], but elevated levels of PAI-1 are also implicated in (components of) cardiovascular disease [37], including vascular inflammation and atherosclerosis. [39] Our current study is not adequately powered to truly establish a possible association between PAI-I, PVAT and PCR, therefore future adequately powered studies should investigate a possible association between PCR and regulation of PAI-I in adipose tissue in the perioperative period, and explore links between PAI-I and potential functional benefits of PCR.

We did not observe any major perturbations in the circulating leukocyte subsets with our PCR intervention, suggesting that these highly evolved cells will be available to participate in the physiologic response to surgical trauma. However, our small sample size limited the conclusions we could draw from our analysis of the innate and adaptive immune system. A larger scale trial that

includes the same leukocyte flow cytometry panel should yield an answer to the question of involvement of the immune system in any potential benefit of the PCR diet.

Previous studies by our group have explicitly linked pre-operative DR with upregulation of endogenous H_2S in endothelial cells [16, 22, 23]. Despite not detecting any upregulation in endogenous H_2S in immune cells as a result of diet or surgery, we did, however, detect remarkable differences in endogenous levels of intra-cellular H_2S between innate and adaptive immune cells, with higher levels in innate cells. Within the innate immune cell groups, there also were strikingly lower H_2S levels in anti-inflammatory monocytes compared to their pro-inflammatory counter parts. Both observations to our knowledge grant a first look into endogenous H_2S immune cell biology, since current knowledge is based on the interaction between exogenous H_2S and innate or adaptive immune cells [40]. Whether specific levels of endogenous H_2S can be directly linked to an immune cells' inherent inflammatory state requires further investigation.

4.1 Conclusion.

The present study provides direct evidence that highlights the safety and feasibility of short-term pre-operative dietary restriction in patients scheduled for elective surgery. Scheduling and logistical issues can make dietary intervention before elective surgery challenging, but PCR is not untenable. As shown here, and previously, both in- and out-patient PCR interventions are feasible and safe, with outpatient trials being a more translational and sustainable intervention as patients incorporate this into their daily lives. In addition the growing body of preclinical and clinical studies on vascular surgery patients, several other studies have shown feasibility and safety of DR in, coronary bypass surgery [41] and patients scheduled for liver resection [42]. Preoperative PCR specifically performed in living kidney donors, in order to enhance recipient kidney function, has previously been shown to be safe and feasible [43]. More recently, a follow-up study pointed towards improved kidney function after transplantation.[44] Excitingly this implicates a role for PCR beyond vascular surgery, which could potentially improve patient outcomes across many fields resulting in better healthcare outcomes and lower costs for patients and hospitals. Future studies should expand upon these in terms of patient recruitment and multi-center trials, as well as by working to validate other preclinical observations such as improved glucose homeostasis[21], wound healing[21] and improved vascular reconstruction durability [22].

4.2 Author Contributions.

Conceptualization, James Mitchell and C. Ozaki; Data curation, Peter Kip, Jodeen Moore, Abby Hart, Jack Ruske, James O' Leary , Jonathan Jung and Michael MacArthur; Formal analysis, Peter Kip, Jodeen Moore, Jonathan Jung, Ming Tao, Michael MacArthur, Patrick Heindel and Alwin de Jong; Funding acquisition, Peter Kip, James Mitchell and C. Ozaki; Methodology, Peter Kip, James Mitchell and C. Ozaki; Project administration, Abby Hart, Jack Ruske and Sarah J. Mitchell; Resources, Jodeen Moore, M.R. de Vries and C. Ozaki; Supervision, Sarah J. Mitchell and James Mitchell; Validation, Ming Tao and Patrick Heindel; Visualization, Alwin de Jong; Writing – original draft, Peter Kip; Writing – review & editing, Ming Tao, Michael MacArthur, Patrick Heindel, Alwin de Jong, M.R. de Vries, M. Furkan Burak, Sarah J. Mitchell, James Mitchell and C. Ozaki.

4.3 Funding.

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4.4 Institutional Review Board Statement.

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Partners Human Research Committee institutional review board, and registered with ClinicalTrials.gov (Identifier: NCT04013412).

Informed Consent Statement. Informed consent was obtained from all subjects involved in the study

Conflict of Interest: none declared

5 . Supplementary.

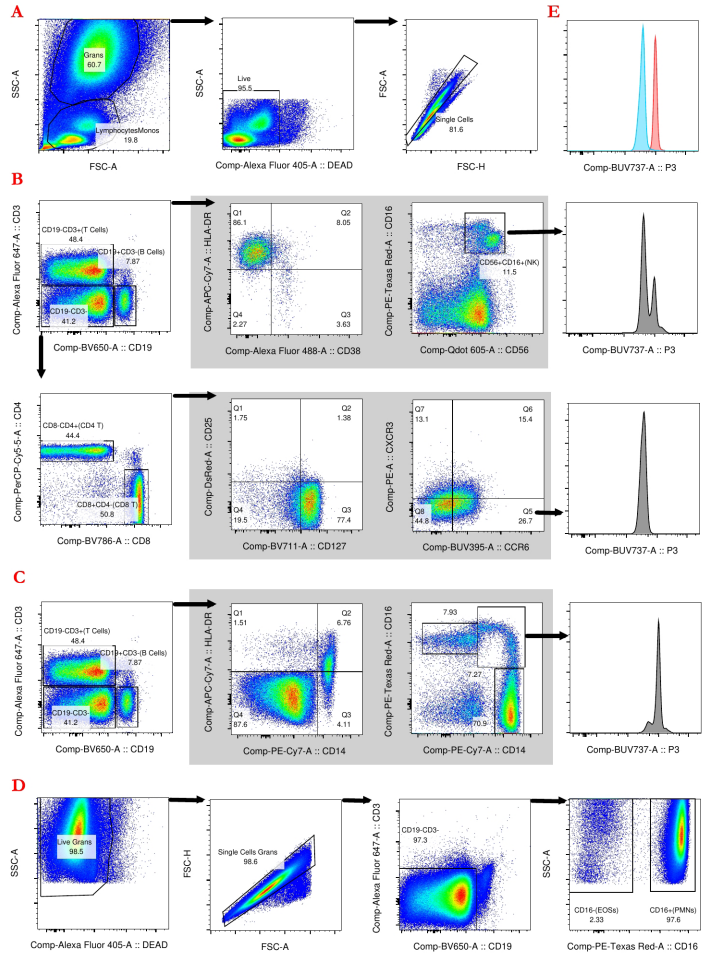
Supplementary Table 1. *Adverse events (AE) at 30 days and one year of follow-up*

	30 Days			1 Year[a]	
Event	**AL, N = 3[b]**	**PCR, N = 8[b]**	**p-value[c]**	**AL, N = 3[b]**	**PCR, N = 8[b]**
Death (All Cause)	0 (0%)	0 (0%)		0 (0%)	1 (12%)
Death (Cardiovascular)	0 (0%)	0 (0%)		0 (0%)	0 (0%)
Stroke	0 (0%)	0 (0%)		0 (0%)	0 (0%)
Myocardial Infarction	0 (0%)	1 (12%)	>0.9	0 (0%)	1 (12%)
Coronary Revasc.	0 (0%)	0 (0%)		0 (0%)	0 (0%)
Conduit Thrombosis	0 (0%)	2 (25%)	>0.9	0 (0%)	2 (25%)
Reintervention	0 (0%)	3 (38%)	0.5	1 (33%)	3 (38%)

[a] 1 year counts inclusive of 30 day counts

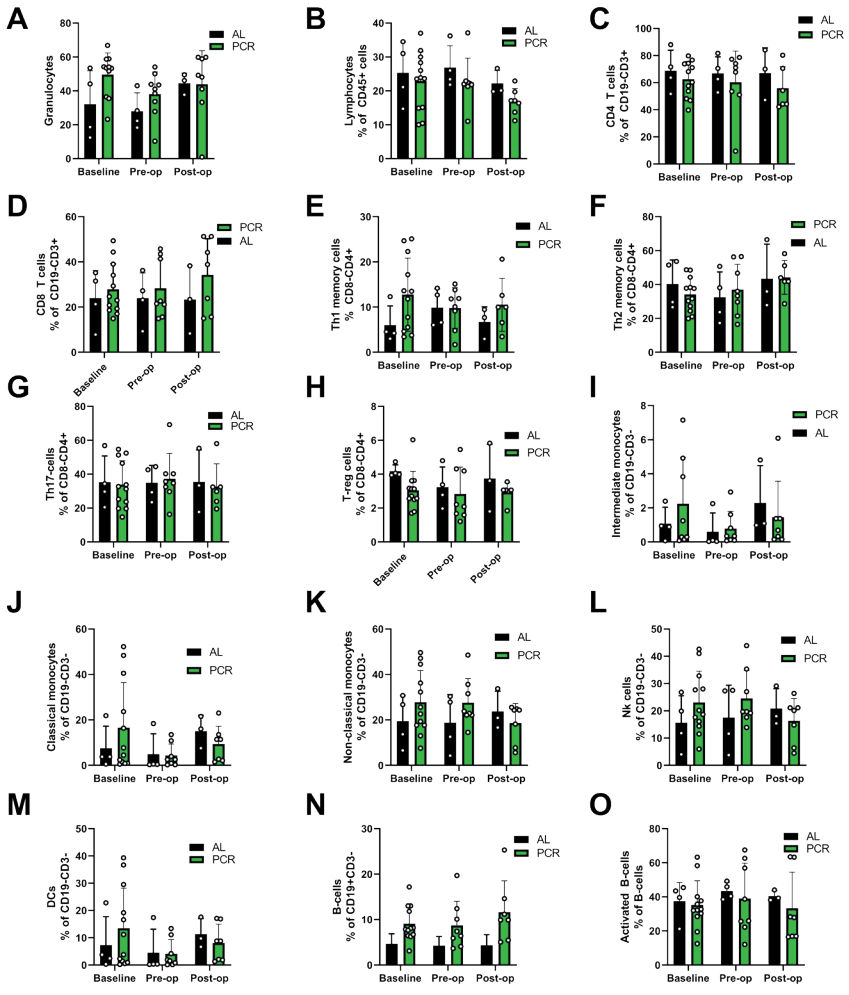
[b] n (%)

[c] Fisher's exact test



Supplementary Figure 1. *Global gating strategy for leukocyte subsets*

A: Via SSC-A and FSC-A granulocyte and lymphocytes/monocytes populations are defined, followed by dead cells exclusion via zombie dye. Via FSC-A and FSC-H single cells are identified. **B:** CD3 and CD19 markers allow for discrimination of T cells (CD3+ CD19-) and B cells (CD3- CD19+). Within the T-cell population, CD4+ and CD8+ T cells, regulatory T cells (CD4+ CD25+ CD127-) and e.g. Th17 cells (CXCR3- CCR6+) can be identified. The MFI for P3 of NK cells has been plotted as an example as well as CD4+ (red) and CD8+ (blue) T-cells. **C:** In CD3- CD19- cells, classical (CD14+ CD16-), intermediate (CD14+ CD16+) and non-classical (CD14- CD16+) monocytes can be identified. In addition, the MFI for P3 of the classical (red), intermediate (green) and non-classical (blue) monocytes is shown. **D:** Gating strategy to identify granulocytes and granulocyte P3 MFI. **E:** P3 MFI of T cells (blue) and classical monocytes (red)



Supplemental Figure 2. *Different subsets of leukocytes at baseline, pre-op and post-op day 1 time-points.*

All subsets are graphed as a percentage of their parent cell population **A:** Granulocytes. **B:** Lymphocytes. **C:** CD4 T-cells. **D:** CD8 T-cells. **E:** Th1 memory cells. **F:** Th2 memory cells. **G:** Th17 cells. **H:** T-regulatory cells. **I:** Intermediate monocytes **J:** Classically activated monocytes. **K:** Non-classical activated monocytes. **L:** Natural Killer cells (NK). **M:** Dendritic cells (DCs). **N:** B-cells. **O:** Activated B-cells

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Chapter 8.

General Summary and Future Perspectives

Atherosclerosis of the coronary and peripheral arteries is a chronic occlusive disease that starts with the formation of a lipid-rich core in the arterial wall. This core will slowly progress towards a necrotic and inflamed atherosclerotic plaque, that is prone to rupture or erosion.[1] Over time patients will develop symptomatology so severe, that a surgical intervention is necessary to prevent myocardial- or peripheral-tissue infarction.[2] For both coronary artery disease (CAD) and peripheral artery disease (PAD), endovascular revascularization or vein graft bypass surgery can be performed to restore blood flow to downstream hypoxic tissues. In general, both coronary and peripheral revascularization strategies, whether endovascular or open bypass surgery, are hampered by high failure rates and frequent peri-operative and post-operative complications.

High incidence of peri-operative stroke/myocardial infarct and post-operative poor wound healing are associated with frequent metabolic disease and other comorbidities in this patient population.[3] Failure of an initial successful procedure however, can be attributed to restenosis/re-occlusion of the vein graft/artery.[4-6] Early failure, i.e. weeks-months post-op, can be attributed to thrombotic occlusion of the conduit. [2, 4] Mid-term failure, which occurs within several months to 1-year post-op, is due to intimal hyperplasia (IH) of the vascular wall.[7] Late-term revascularization failure can be attributed to re-occurrence of an atherosclerotic lesion within the revascularized vessel wall.[8] For both mid-term and late-term failure after revascularization currently no treatment exists[9, 10], resulting in high re-admission and re-intervention rates.[11] This unmet clinical need amounts to an incredible burden of suffering and death for patients and families, while also putting enormous strain on the healthcare system[12, 13], warranting new strategies to impair arterial IH and VGD.

The aim of this thesis was to examine the potential of short-term dietary restriction (DR) in (cardio)vascular surgery, both in preclinical rodent models of IH and vein graft disease as well as vascular surgery patients. DR comes in various forms and comprises a reduction or change of either total calories, total proteins, specific amino-acids or a combination. Long-term (months-years) DR is best known for its ability to extend lifespan in rodents, but also mitigates post-operative injury in preclinical models of ischemia reperfusion injury in the heart and brain.[14, 15] In clinical practice however, in the setting of acute stress such as planned surgery, long-term DR is not feasible and impractical. It was Mitchell and colleagues[16] who adapted this concept of long-term DR towards a practical short-term diet, and discovered that a brief (days-week) reduction in calories or proteins before undergoing surgery is sufficient to protect from surgical ischemia reperfusion injury in kidney and liver. Here, we built on that initial finding and tested different forms of DR in several rodent models of (cardio)vascular surgery.

Chapter 2 first interrogated the effects of short-term DR on wound healing in both non-diabetic and diabetic mice. Previous research found an association between severe PR and delayed wound healing[17], but this concerned a long-term (12-week) diet. In this study, we tested a short-term PR and a short-term methionine restriction (MetR) diet in an established model of wound healing: the McFarlane flap model.[18] Compared to control-fed mice, both MetR and PR preserved wound healing potential. Peri-operative fasted glucose levels were lower, up to 3 days post-surgery in both diet groups, while glucose tolerance was also improved. Dysregulated glucose levels pre-, peri- and post-surgery have long been associated with poor wound healing.[19] Moreover, peri-operative hyperglycemia has also been linked with poor outcome after lower extremity bypass surgery, correlating with increased mortality and revascularization failure.[20] Here we show that short-term pre-operative PR or MetR does not interfere with the wound healing response, since wound closure was not hampered. PR/MetR also improved post-operative hyperglycemia in diabetic mice, indicative of a potential role for these dietary interventions in the management of peri-operative glucose homeostasis. Recent work suggests that one mechanism of action for this endocrine effect could transpire through upregulation of fibroblast-growth-factor 21 (FGF-21)[21], although likely several pathways are involved.

In **chapter 3** we tested the potential of short-term preoperative protein restriction (PR) in a rodent vein graft surgery model. Previously, Hine et al.[22] discovered that short-term DR protects from ischemia reperfusion injury in the liver via increased production of endogenous hydrogen sulfide (H_2S). In their study, they found that a short-term reduction in calories (CR) upregulated cystathionine y-lyase (CGL), the main H_2S producing enzyme in the vasculature.[23] We discovered that short-term PR also increases endogenous H_2S production, specifically in aorta and caval vein endothelial cells (ECs). Functionally, short-term PR protected from vein graft disease in an established model of vein graft surgery. Mechanistically, PR upregulated the CGL enzyme both in the vessel wall and ECs of the vessel wall, and benefits of PR were lost after pharmaceutical blocking of CGL activity. Genetic upregulation of CGL, via a novel overexpressing mouse model, also yielded protection from graft

failure. Both dietary (via PR) increased production- and genetic overexpression of CGL resulted in limited vascular smooth muscle cell (VSMC) migration and neutrophil transmigration. Here we establish a novel approach to attenuate IH and vein graft disease in a validated rodent model of vein bypass surgery. With only a 7-day isocaloric reduction in total proteins before undergoing vein graft surgery, we were able to limit the intimal hyperplastic and fibroproliferative response in the vein graft wall. Combining this study's results with **chapter 2**, we can now hypothesize that DR is not only capable of improve perioperative metabolic fitness but also impact long-term functional outcome after vein graft surgery by increasing conduit performance and durability.

In **chapter 4**, we tested the effectiveness of short-term MetR in both a model of arterial injury, via carotid artery focal stenosis creation, and vein graft interposition grafting. MetR limited IH and improved remodeling in both models of revascularization failure, and the effect-size was comparable with vein grafts after PR. A pro-inflammatory perivascular adipose tissue (PVAT) phenotype at the time of surgery has been linked to adverse outcome, while "sick" PVAT was found to be predictive of future wound complications.[24, 25] In preclinical models, obesity-induced "sick" PVAT accelerates neo-intima formation[26], while the response to surgical trauma in adipose itself is also exacerbated in the context of this phenotype.[27] Long-term MetR has been shown to confer an anti-inflammatory phenotype in inguinal adipose tissues[28], but its effect on PVAT is unclear.

We discovered that MetR protects from vein graft remodeling via an interaction between the diet and the presence of PVAT surrounding the vein graft. The dietary intervention modulated caval vein PVAT phenotype from having a detrimental- towards a protective impact on vein graft remodeling, possibly through AMPK-activation and increased thermogenesis. Transcriptomic analysis of both caval vein (venous) and thoracic aorta (arterial) PVAT revealed distinct adipose phenotypes between the two depots. But after one week of MetR, caval vein PVAT more closely resembled arterial (thoracic aorta) in its transcriptomic profile. At post-op day 1 (POD1), PVAT of vein grafts who were preconditioned with MetR showed a significantly dampened pro-inflammatory and -atherosclerotic response compared to their control-fed counterparts.

As an alternative to dietary preconditioning to improve vascular remodeling, we developed a DR-mimetic that could be applied locally and periprocedural. **Chapter 5** concerns the development and testing of this replacement strategy

for short-term DR. Since the benefits of DR in surgical stress models are partly dependent on upregulation of endogenous H_2S[22], we aimed to developed a Pluronic gel containing a H_2S-releasing prodrug. This therapeutic strategy to deliver exogenous H_2S local and periprocedural was able to attenuate IH and improve vein graft remodeling, functioning as an effective alternative for endogenous H_2S upregulation (via short-term DR). Mechanistically we found that part of this beneficial effect on vein graft remodeling was accomplished by impaired VSMC migration via the H_2S-prodrug. In short, we developed a strategy to directly deliver exogenous H_2S onto the vessel wall that was able to improve arterial and vein graft remodeling. This DR-mimetic offers an alternative to dietary preconditioning and broadens the therapeutic potential of this concept.

Next to our work in preclinical models, we extended our studies to clinical application. We tested the practicality and feasibility of short-term DR in human subjects. In **chapter 6** we enrolled and randomized patients scheduled for elective carotid endarterectomy on either a 3-day PCR diet or an *ad libitum* (AL) diet before undergoing surgery. To closely monitor patient-safety, especially in the context of metabolic disease, we asked patients to reside in the surgical ward during the course of the dietary intervention. Although we only enrolled 4 patients, which was most likely due to the in-patient requirement pre-op, we were able to make some interesting observations. Sequencing of baseline, pre-op and POD1 stool samples revealed an intriguing shift towards abundance of the H_2S-producing bacterial species *Bilophila wadsworthia*[29] when comparing pre-PCR with post-PCR stool samples.

In **chapter 7** we extended our clinical study by extending the inclusion criteria towards any patient scheduled for any elective vascular surgery operation that involved open surgical wound creation. Furthermore, since no adverse- or compliance events were detected in our previous pilot study, we extended the course of the diet from 3- to a 4-day PCR diet, which patients were able to ingest at home. In this randomized clinical controlled trial, we were able to enroll 19 patients in this study, of which 10 completed the study. Unfortunately, due the rapidly emerging covid-19 pandemic, we were forced to prematurely halt patient-enrollment to this trial. Nonetheless, although underpowered, we were able to detect some interesting diet-effects. BMI in the pre-operative PCR group tended to be lower compared their pre-diet levels, indicating diet-compliance. A relative decrease in fasted insulin compared to baseline levels was also seen in the PCR group, which coincides with the effects of long-term MetR in humans.[30] Also at the pre-op time-point, the PCR group had a significant relative increase

in circulating basophils compared to AL-fed humans. An although the precise role of this leukocyte-subset in (cardio)vascular surgery is unknown, it has been implicated in the regulation of both the innate and adaptive immune response.[31] Ongoing analysis of an extensive leukocyte flow cytometry-panel in the peripheral blood of these patients, which is combined with an intracellular H_2S-probe, will hopefully allow us to re-iterate or expand on this finding.

Most importantly, the trial demonstrates that short-term PCR in vascular surgery patients is both feasible and practical. None of the enrolled subjects who failed to complete the trial did so due to adverse effects of the diet, nor were there any detectable compliance issues.

Future Perspectives.

This thesis demonstrates that short-term DR is effective in mitigating the intimal hyperplastic response to vascular injury, both after an arterial intervention and vein graft surgery. Via a brief reduction in either total protein or specific amino acids, we were able to significantly improve vascular remodeling. Next to these protective benefits linked to revascularization success, peri-operative glucose homeostasis was also enhanced, which could extend the purpose of these dietary interventions beyond (cardio)vascular surgery to other surgical disciplines. Common guidelines for the peri-operative management of patient nutrition are described in the Enhanced Recovery After Surgery (ERAS) protocols[32], and implementation of ERAS in vascular surgery patients is linked with shorter in-hospital stays.[33] The nutritional recommendations in these protocols mostly concern with optimal and immediate post-operative re-feeding, while an optimal pre-surgery feeding state is described as a "bodily state without malnutrition or malnourishment". Recommendations for ERAS also include ingestion of a carbohydrate drink ("carbohydrate loading") in the 24 hours before surgery to avoid a fasted state, and this is linked with improved glucose homeostasis.[34] In this thesis, we present our case for an alternative view on pre-operative feeding and dietary advices, which should not need to interfere with established (post-operative) ERAS principles.

Preclinically we demonstrated a novel method to improve glucose balance that only concerns a short-term pre-operative reduction in either proteins or specific amino acids, which are replaced with carbohydrates to maintain caloric value. ERAS protocol principles and short-term DR are therefore not mutually exclusive, since carbohydrate loading shortly before surgery does not interfere with protein or methionine restriction in the days leading up to the surgery.

Immediately post-op ERAS recommendations can still be implemented, since our strategy only concerns a pre-operative dietary intervention. Both our clinical trials concerned a combination of protein and calorie restriction in the days leading up to the surgery. Daily intake was calculated by qualified dieticians to prevent malnutrition, nor were patients fasted outside the required pre-operative 8 hours. Whether diabetes was present or not, none of the patients on a PCR diet experienced any hyper- or hypoglycemic adverse effects. An adequately powered future clinical trial of short-term PCR in (cardio)vascular surgery patients should implement a secondary endpoint concerning glucose homeostasis in the pre- and peri-operative window. Frequent measurements of glucose in both diabetic and non-diabetic patients from both dietary arms of the study would hopefully reinforce our findings in rodent models. This could show additional benefits beyond revascularization success, since adequate peri-operative glycemic control is clearly linked with post-operative complications and wound healing.[19, 35]

Next to peri-operative health improvements, this thesis mainly focused on the benefits of short-term DR in vascular wall remodeling. As a first proof of principle, we tested a short-term total reduction in proteins in vein graft surgery. Just one week of PR was able to attenuate vein graft disease, and these benefits were partly derived from endogenous H_2S upregulation. In a follow-up study we investigated the potential of short-term restriction of specific amino acids, methionine and cysteine, in vascular remodeling and were able to specifically show a PVAT dependent mechanism of action. Interestingly, several clinical studies have been conducted that investigated the benefits of "no touch" harvesting of the saphenous vein for coronary artery bypass surgery. This technique aims to harvest the vein en-bloc with surrounding vasa vasorum and PVAT intact.[36] Although initial prospective studies were promising, a recent randomized clinical trial failed to show benefits in vein graft patency.[37] Our preclinical studies in the interaction between MetR and vein graft PVAT indicated that our control group with intact vein graft PVAT had an exacerbated remodeling phenotype. This disadvantageous effect of PVAT on intimal hyperplasia is known from other preclinical studies where PVAT from obese mice accelerated neointimal hyperplasia.[38] Our dietary intervention however, was able to modulate the role of PVAT from detrimental to beneficial. Just one-week of MetR improved vein graft remodeling specifically via PVAT, suggesting a reversal of PVAT-phenotype from "sick" to healthy. In this recently conducted and failed "no-touch" trial, both patient groups had a body-mass index of 28-29[37], which is to be expected in a cardiovascular patient population. However,

an on average overweight patient population could also indicate the presence of obesity-induced "sick" PVAT in a subsection of these patients, which would explain the absence of any treatment benefits.

Mechanistically, our findings indicate that MetR activates AMPK-signaling in the PVAT, and future preclinical studies should focus on the specific local effects of AMPK regulation in vein grafts. Either dietary (via MetR) or local pharmacological therapies with AMPK activators could modulate PVAT phenotype towards protection from graft remodeling and our findings certainly warrant further studies into the specific cell types involved. Semi-synthetic MetR diets in humans are feasible, with proven metabolic and adipose-specific health benefits.[39] Our discovery also favors a second look into the "no-touch" principle in bypass surgery, but then in the context of dietary preconditioning. A clinical study in conventional and "no-touch" harvested grafts from patients on short-term pre-operative MetR or semi-synthetic control diets, would allow us to look for our preclinically established biomarkers of "healthy" PVAT. A first study should focus on periprocedural sampling of adipose tissue during vein harvest, and PVAT transcriptome should be interrogated for AMPK activation and inflammatory status, before undertaking a large trial with graft patency endpoints.

Although we did not specifically search for endogenous H_2S upregulation after MetR, we were able to link benefits from PR to an increase in endogenous H_2S. As of today, short-term DR is the only known intervention to increase endogenous H_2S[40], but a dietary preconditioning strategy is not always feasible. Whether due to issues with diet compliance or the preconditioning time available before undergoing surgery, alternative strategies to mimic DR should be explored. One candidate for a DR-mimetic is exogenous H_2S therapy, and this has indeed been proven effective in mitigating cardiovascular disease and injury. And although several drugs are currently in early phase clinical trials, these therapies usually involve systemic administration of H_2S over multiple days/weeks.[41] Here we present an alternative to systemic administration of H_2S, with its associated potential toxicity and adverse reaction issues, via a one-time local administration of a H_2S-releasing gel directly on the vascular wall. Our local gel attenuated both arterial intimal hyperplasia and vein graft disease, which indicates efficacy in the prevention of endovascular neo-intimal hyperplasia. Future collaborations, with an academic- or industry-based group holding expertise in biomaterials development and optimization, could yield additional applications and benefits.

Firstly, a follow-up study in a rat model of balloon-angioplasty could explore whether subcutaneous injection of this gel into the perivascular sheath of the artery yields similar protection from neo-intimal hyperplasia. Secondly, its usefulness in arteriovenous fistula maturation should be investigated, since currently no therapy exists to prevent non-maturation. In such a rodent fistula model, next to periprocedural application, post-operative injection with the H_2S-gel should also be tested as a treatment option to recover non-maturated fistulas. Lastly, although exogenous H_2S therapy already has been linked to accelerated wound healing[42, 43], these studies were conducted with fast-releasing H_2S donors/drugs as opposed to our slow- and extended-release substance. Sustained H_2S release onto the wound bed could yield additional benefits throughout the wound healing process. Especially in vascular patients, such a sustained H_2S therapy could prevent progression towards a chronic non-healing ulcer as is frequently seen in this patient subpopulation.[44]

Ultimately, the goal here is to work towards large-scale clinical trials that are adequately powered to study the effectiveness of such a slow-releasing H_2S gel when applied perioperatively/procedurally on primary patency after vein graft and endovascular interventions.

Finally, in this thesis, we conducted two randomized controlled clinical trials with short-term DR in vascular surgery patients. Since preclinical work showed additional benefits of protein combined with calorie restriction, we opted for a protein-calorie restriction (PCR) diet. Our first study concerned an in-patient setting to adequately monitor patient-safety and diet-effects. Although we only enrolled four patients, all randomized into the PCR group, our findings in their microbiome yielded some insights in H_2S biology in humans. Specifically, stool from patients before and after 3-days of PCR indicated an increased abundance of the sulfide producing bacterial species *Bilophila wadsworthia*[29] Our follow-up study in vascular surgery patients in an out-patient setting was more successful in enrolling study subjects, since patients were not required to prolong their total time spent admitted to the surgical ward. Although our enrollment was shortened due to the covid-19 outbreak, over the course of 1.5 years only 19 patients consented to the study. Successful study recruitment is likely dependent on several factors, and the unfamiliarity of patients with this novel concept could play a role together with the challenge of installing dietary intake changes in a study population that is prone to lifestyle diseases.

Future steps to build and expand on this most recent clinical trial in vascular surgery and DR should encompass a multi-arm strategy that aims to define the correct diet that is both feasible and safe in this challenging patient population. Currently several grant proposals and clinical trials are in our study group pipeline, which include short-term DR in patients scheduled for arteriovenous fistula surgery and time-restricted feeding in patients planned for open femoral endarterectomy. The latter encompassing an alternative route to similar metabolic benefits seen as in DR[45], not via restricting a certain macro-nutrient but by limiting the window per day for patients to ingest their regular diet. Perhaps this strategy could reinforce patient participation willingness since there is no quantitative or qualitative alteration in the patient's diet.

In conclusion, in this thesis we interrogated the benefits of short-term DR in (cardio)vascular surgery and vascular remodeling. A short-term reduction in proteins or amino acids is effective in mitigating the surgical and vascular response to injury, both via improved perioperative glucose homeostasis and a favorable vascular remodeling phenotype. Short-term DR increased endogenous levels of H_2S, while a DR-mimetic was able to deliver exogenous H_2S locally and sufficiently. Both endogenous and exogenous H_2S upregulation stands as effective strategies to mitigate revascularization failure and warrant future studies in other (vascular) surgery models. The finding that diet favorably modulates adipose tissue phenotype is another underlying mechanism of action with enormous potential beyond vein graft surgery, since essentially all surgical procedures involve adipose tissue manipulation. In vascular surgery patients, short-term DR is feasible and safe, and we gained valuable insights in the response in surgical patients to dietary preconditioning.

To summarize, this thesis can serve as a preclinical foundation and early clinical basis for our current and upcoming clinical trials. Our long-term efforts should focus on adequately powered clinical trials to rigorously test the hypothesis that short-term DR can improve vascular conduit patency. Ultimately, we envision a vascular surgery clinical practice where every patient scheduled for elective vascular surgery is first subjected to either a preoperative DR regimen or application of a perioperative DR-mimetic when DR is not feasible or suitable. Both strategies should ensure maximum perioperative and post-operative benefits for this frail and sick patient population.

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Nederlandse Samenvatting

Aderverkalking van de coronair en perifere bloedvaten is een chronische ziekte welke ontstaat door schade aan de beschermde binnenste laag van de vaatwand. Door roken, ongezond eten, hoge bloeddruk en suikerziekte ontstaat een gat in deze beschermende laag wat tot gevolg heeft dat circulerende ontstekingscellen de vaatwand in kunnen en een ontstekingsproces op gang brengen. Uiteindelijk kan dit onstekingsproces tot gevolg hebben dat de wand van het bloedvat naar binnen groeit en de toevoer van bloed gedeeltelijk of geheel wordt gestopt. Dit resulteert in verminderde of geheel afwezige toevoer van zuurstof en voedingsstoffen in het omringende weefsel. Wanneer dit in de bloedvaten rond het hart plaatsvindt resulteert dit in een hartaanval en wanneer dit in de benen gebeurt leidt dit tot forse pijnklachten in het been en verminderede loopafstand of zelfs een afstervend ledemaat.

Wanneer gezonder leven en bepaalde medicijnen niet voldoende helpen tegen deze klachten, is de enige goede oplossing een chirurgische operatie. De eerste voorkeur heeft een zogenaamde “endovasculaire” ingreep, ook wel “dotteren” genoemd. Wanneer dit niet mogelijk is wordt met een stukje gezond bloedvat een omleiding om het dichtzittende deel van het bloedvat gelegd, een zogenaamde “bypass” operatie. Hierbij wordt de bloedtoevoer naar het achterliggende weefsel weer hersteld en krijgt het desbetreffende lichaamsgedeelte weer voldoende zuurstof en voedingsstoffen. Deze bypassoperatie heeft op de korte termijn goede resultaten, echter zie je vaak dat deze bloedvaten op termijn ook weer dicht gaan zitten door een ziekteproces dat vergelijkbaar is met de zojuist beschreven aderverkalking, dit wordt “veneuze graft ziekte” genoemd. Therapieën om veneuze graft ziekte af te remmen of te stoppen zijn momenteel niet voorhanden.

Al in 1937 bleek uit een experiment uitgevoerd met ratten dat wanneer je de totale hoeveelheid dagelijkse calorieën in het dieet verminderd met ongeveer 20-30%, deze ratten langer leven dan ratten die zoveel konden eten als ze wilden. Dit principe van “dietary restriction”, “dieet restrictie” of DR werd in dit experiment toegepast vanaf de geboorte tot hun dood. In vervolgstudies met DR in allerlei organismen, van wormen tot muizen tot katten tot apen, is dit effect van een levenslange vermindering van dagelijkse calorie intake aangetoond op een verlenging van de levensverwachting.

In 2010 besloot dr. James R. Mitchell om dit principe van DR toe te passen op de fase in aanloop naar een chirurgische operatie, de zogenaamde “preoperatieve” fase. Hij besloot om 4 weken voor de start van de operatie muizen op zo`n DR-dieet te zetten, de operatie uit te voeren en ze meteen weer hun normale “controle” dieet te laten hervatten dat precies dezelfde hoeveelheid calorieën bevat als voordat ze op hun DR-dieet werden gezet. De operatie bestond uit het kortdurend klemmen van beide nier-arteriën en daarna de klem weer te verwijderen, om ischemie-reperfusie schade na te bootsen dat optreedt bij orgaantransplantaties en verscheidenen vaatchirurgische operaties. In z`n experiment bleek dat de muizen die in de 4 weken voorafgaande aan de operatie een DR-dieet hadden gekregen een verbeterde overleving hadden na de operatie, ook werd er minder schade aan de nier gemeten, in vergelijking met muizen die een normaal dieet hadden gekregen preoperatief.

In verscheidene follow-up studies heeft hij met z`n onderzoeksgroep uitgevonden dat een groot gedeelte van de effecten van deze DR-diëten op chirurgische uitkomsten worden veroorzaakt door versterkte lichaamseigen productie van waterstofsulfide ($H_2S$). Dit gasvormig molecuul, met z`n kenmerkende “rotte eieren” geur, heeft als voornaamste functie om de bloeddruk te reguleren en de bloedvaten te verwijden als de fysiologische situatie in het lichaam hierom vraagt. Nu blijkt dat dit gas ook wordt geproduceerd in reactie op een DR-dieet, en het dan verscheidene negatieve effecten van een chirurgische ingreep remt. Zo vermindert het de ontstekingsreactie en beschermt het cellen tegen celdood.

In mijn huidige proefschrift heb ik onderzocht wat de effecten zijn van zo`n DR-dieet in vaatchirurgische operaties. In **hoofdstuk 2** heb ik eerst bestudeerd wat het effect van zo`n preoperatief DR-dieet is op de postoperatieve wondgenezing is. Wanneer we een eiwit-restrictie dieet (dus een dieet waarbij de eiwitten zijn vervangen door koolhydraten, PR) vergeleken met een normaal dieet zagen we geen verschil in wondgenezing. Wanneer we een methionine-restrictie dieet (de hoeveelheid methionine aminozuur verminderen en vervangen door koolhydraten, MetR) toepasten in de preoperatieve periode, zagen we postoperatief ook geen verminderde wondgenezing. In zowel het PR als het MetR zagen we wel een verbeterde regulatie van de bloedsuiker vergeleken met het controle dieet. Uit eerder onderzoek is gebleken dat slecht gereguleerde bloedsuikers een voorspeller kunnen zijn voor slechte wondgenezing na een operatie.

In hoofdstuk 3 hebben we gekeken naar de effecten van PR op veneuze graft ziekte in muizen. We hebben in die studie gezien dat je maar 1 week preoperatief muizen op een PR-dieet hoeft te zetten, en dat dan 4 weken na de

operatie er veel minder veneuze graft ziekte is in vergelijking met muizen die voor de operatie een normaal dieet mochten eten. Dit effect werd veroorzaakt door toegenomen productie van een van de enzymen die H_2S produceert in het lichaam.

In hoofdstuk 4 hebben we wat specifieker gekeken welk gedeelte van de veneuze graft wand het meeste effect van het dieet ondervindt. De veneuze graft bestaat uit een binnenste intimale wand, een mediale wand en een buitenste adventitia wand. Ook bevindt zich rondom de graft nog een laag vetweefsel, het zogenaamde perivasculaire vet (PVAT). Dit vet bevat zeer veel signaal-moleculen en factoren die een forse invloed hebben op de onderliggende vaatwand. In ons experiment hebben we de helft van de veneuze grafts gestript van het PVAT voordat we ze in de muis aanlegden. De andere helft hield als z`n PVAT. In beide cohorten was de ene helft eerst een week op MetR gezet, de andere helft mocht het controle dieet eten. Vier weken na de operatie bleek dat de muizen die preoperatief MetR hadden gekregen en vervolgens het PVAT rondom hun veneuze graft hadden behouden de minste veneuze graft ziekte hadden. De muizen op een controle dieet met het PVAT nog intact hadden de meest hevige vorm van veneuze graft ziekte. In beide dieet-groepen zonder PVAT was er geen meetbaar verschil. Anders gezegd, we hebben ontdekt dat er een specifieke interactie bestaat tussen het MetR-dieet en het PVAT, en dat je deze interactie nodig hebt om een positief effect op veneuze graft ziekte te zien.

In hoofdstuk 5 hebben we gekeken wat er gebeurt als je direct H_2S op de veneuze graft aanbrengt in plaats van de productie daarvan op te wekken via een preoperatief dieet. Door een medicijn dat H_2S bevat op te lossen in een zogenaamde “pluronic gel”, een gel die op 4°C vloeibaar is maar op kamertemperatuur stolt, konden we de veneuze graft tijdens de operatie omvatten met een soort deken van H_2S deeltjes. 4 weken na de operatie bleek dat de groep die de gel + H_2S had gekregen in plaats van de gel + een placebo een veneuze graft had met een veel grotere diameter en een gezondere vaatwand.

In hoofdstuk 6 en 7 hebben we ons preklinische werk in muismodellen om kunnen zetten naar twee klinische studies met vaatchirurgische patiënten. De eerste trial in **hoofdstuk 6** betrof patiënten die waren gepland voor een halsslagader desobstructie operatie, waarbij ze werden gerandomiseerd in of 3 dagen protein-calorie restriction (zowel restrictie van eiwitten als calorieën, PCR) of hun normale dieet. Gezien het de eerste pilotstudie ooit betrof in deze patiëntenpopulatie hebben we ze gedurende het dieet in het ziekenhuis laten verblijven om ze goed te kunnen observeren. Er werden geen negatieve effecten van het PCR-dieet geobserveerd in onze studie-patiënten, maar de hoeveelheid geworven patiënten was te laag om een uitspraak over dieet-effecten te kunnen doen. Wel zagen we een toename van H_2S producerende bacteriën in de ontlasting van patiënten die het dieet net achter de rug hadden. In **hoofdstuk 7** hebben we patiënten geworven die voor een willekeurige open vaatchirurgische operatie gepland stonden en het dieet verlengd met 1 dag tot 4 dagen PCR preoperatief. Patiënten hoefden tijdens het dieet niet meer in het ziekenhuis te verblijven. Helaas moest de werving van patiënten gestopt worden vanwege het uitbreken van de COVID-19 pandemie maar desalniettemin zagen we een kleine verbetering in de bloedsuiker regulatie, ondanks het relatief kleine aantal patiënten.

Concluderend, in dit proefschrift hebben we de werkzaamheid van preoperatieve DR-diëten aan kunnen tonen. Specifiek hebben we laten zien dat het veneuze graft ziekte verminderd, en dat dit waarschijnlijk afhankelijk is van de interactie tussen het dieet en het PVAT. Verder hebben we laten zien dat je het dieet ook kunt vervangen door een peroperatieve toediening van H_2S, om zo de dieet effecten op de veneuze graft na te bootsen. Verder hebben we tevens een tweetal klinische trials uit kunnen voeren in vaatchirurgische patiënten, waarbij we hebben kunnen vaststellen dat het dieet veilig is en ervaring op hebben kunnen doen met het organiseren van dergelijke dieet-restrictie trials in de vaatchirurgische patiëntenpopulatie.

In de toekomst zullen we ons op preklinisch vlak richten op het verder uitdiepen van de relatie tussen het PVAT en het dieet, om te kijken of we dit effect verder kunnen versterken dan wel nabootsen met een medicijn. Op het klinische vlak richten we ons op het optimaliseren van het meest geschikte dieet dat therapietrouw combineert met effectiviteit. In de niet-zo-verre toekomst bestaat er hopelijk een vaatchirurgische praktijk waarbij elke patiënt een preoperatief dieet tot zich zal nemen, of peroperatief een middel krijgt toegediend dat de effecten van DR nabootst, of beide. Hopelijk legt dit proefschrift de basis voor een dergelijk klinisch toekomstbeeld.

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Curriculum vitae

The author of this thesis was born on the ninth of May of 1989 in Emmen, the Netherlands. He grew up in a little border town called “Zwartemeer” in Drenthe. In the city of Emmen he first completed HAVO in 2005 followed by VWO (pre-university) in 2007. Until 2008 he played handball on a (semi)-professional level at E&O. After graduating VWO he studied Life Science and Technology in Groningen, before he was allowed into the Leiden University Medical School in 2009. During his medicine school studies he joined the lab of Professor Paul Quax and Margreet de Vries for extracurricular work on endarterectomy plaques. After graduating in 2017, he started his PhD in the lab of Paul and Margreet, but shortly afterwards moved to Boston to the labs of Prof. Ozaki (department of Vascular and Endovascular Surgery, Brigham and Women’s Hospital/Harvard Medical School) and Prof. Jay Mitchell (Department of Molecular Metabolism, Harvard T. H. Chan School of Public Health). Here he spent the next three years working on pre-operative dietary restriction in vascular surgery preclinical mouse models and patients. In August of 2020, he moved back to the Netherlands and entered a position as surgical resident (not-in-training) in the Alrijne hospital Leiderdorp. In January 2022 he started his training program as a surgical resident in training in Leiden.

List of Publications

Kip P, Sluiter TJ, Trocha KM, MacArthur MR, Tao M, Mitchell SJ, Jung J, Quax PHA, Mitchell JR, Ozaki CK, de Vries MR (2020). Short-term methionine restriction protects from vein graft disease via perivascular adipose tissue dependent AMPK-signaling. *In progress*

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Grants

American Heart Association Post-Doctoral Research Fellowship. Post-doctoral Fellowship for research proposal titled: *"Vasculoprotective Mechanisms of Endogenous Hydrogen Sulfide in Vein Grafts"*. $104.060 for the period of 01/2019 – 12/2020 at the Brighamd and Women's Hospital and Harvard Medical School. (ranked in percentilel 0.17%, priority score 1.31)

Michael-van Vloten Fonds. *"Kortdurende Preoperatieve Eiwitrestrictie ter Preventie van Veneuze Bypass Falen"*. €8.000; for the period of 2019-2020 at the Brighamd and Women's Hospital and Harvard Medical School

Prins Bernhard Fonds. "*Eiwitrestrictie in Veneuze Graft Ziekte"*. €10.000; van 2018-2020 at the Brighamd and Women's Hospital and Harvard Medical School.

Stichting de Drie Lichten. "*De Effecten van Sulfaat Aminozuur Restrictie en Waterstofsulfide op Post-interventionele Inflammatie in Veneuze Grafts"*. €12.000; van 2017-2019 at the Brighamd and Women's Hospital and Harvard Medical School **LUF-fonds.** "*Kortdurende Eiwit Restrictie ter Verbetering van de Uitkomsten na Veneuze Bypass Chirurgie"*. €1000

Awards

Finalist fo the 2019 Stepping Strong Innovator Award. Finalist with proposal and elevator pitch titled: *"Hydrogen Sulfide Gels to Accelerate Traumatic Wound Healing."* For the Brigham and Women's Hospital Gillian Reny Stepping Strong Center for Trauma Innovation.

BWH Department of Surgery John A. Mannick Research Day Award 2019. Best abstract *"Endogenous and Exogenous Hydrogen Sulfide Upregulation as Novel Strategies to Improve Vein Graft Durability.* $400 award and oral presentation.

Vascular Research Initiatives Conference Trainee Award 2019. Best abstract titled *"Peri-procedural Local Hydrogen Sulfide Therapy Impairs Vascular Remodeling and Improves Vein Graft Patency."* Award and oral presentation

8th Annual Harvard-Longwood Surgical Research Day Award 2019. Best abstract titled: "*Peri-procedural Local Hydrogen Sulfide Therapy Impairs Vascular Remodeling and Improves Vein Graft Patency."* Award and oral presentation

BWH Peripheral Artery Disease Symposium 2018. Top 3 ranked abstract titeld: *"Hydrogen Sulfide Improves Vein Graft Patency and Limits Intimal Hyperplasia".* $600 award and oral presentation

North American Vascular Biology Organization Travel Award 2018. $250 award and poster-presentation

ATVB/PVD Travel Award 2018. $1000 award and (poster) presentation

Society of Vascular Surgery Resident Research Award for Best Manuscript 2017-2018. *"Pre-Operative Protein-Restriction Attenuates Vein Graft Disease Via Induction of Cystathionine gamma-lyase"* Trocha K*, **Kip P*** et al. $5000

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Kip P. Moore J. Hart A. Ruske J, O'Leary J, Sluijter TJ, Jung J, Tao M, MacArthur MR, Mitchell SJ, Mitchell JR & Ozaki C Keith. *Short-term Pre-operative Protein Caloric Restriction in Elective Vascular Surgery Patients: A Randomized Clinical Trial*. 2nd Protein-calorie restriction workshop: Short-term Dietary Interventions to Improve Surgical Outcomes and Beyond (Oktober 2020); online conference. *Oral*

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