

DIETARY RESTRICTION IN VASCULAR SURGERY

Key Unmet Need Addressed

Vascular surgery continues to offer enormous benefits to selected patients. However, vascular patients continue to suffer some of the highest complications rates: cardiac morbidity (e.g. myocardial infarction [MI]), stroke, ischemia/reperfusion injury (IR), restenosis, and death peri-operatively, with subsequent high expense for our health care systems and enormous toll for the individual patients¹⁻³. With the exception of a few evidence-based guidelines that modestly improve perioperative care, there is no uniformly accepted medical therapy to reduce complications in these patients⁴. Rising health care expenditures, the aging population, and the increasing incidence of the metabolic syndrome all point to a dire need for accelerated research into feasible strategies to enhance outcomes of major operations⁵.

Project Goals/Specific Aims

Hydrogen sulfide (H₂S) has emerged as a critical gaseous signaling molecule with a role in multiple processes including IR injury, angiogenesis, intimal hyperplasia, and anti-inflammatory mechanisms⁶⁻¹¹. It even appears to hold anti-atherosclerotic properties¹². However, the gas is toxic with a half-life of minutes, and it can be rapidly oxidized. Furthermore, no clinically useful pharmacologic H₂S donors have been developed to date. In our 2015 *Cell* paper, we link substantial upregulation of endogenous H₂S to short-term manipulation of mammalian dietary intake: simple dietary restriction¹¹. The current project serves as the second phase in the accelerated translation of this important discovery toward clinical utility that may substantively impact the vascular patient at several levels: protection from IR injury, intimal hyperplasia, peri-procedural events such as stroke, cardiac dysfunction and MI, and promotion of angiogenesis^{7, 8, 12-17}.

Chronic protein and calorie dietary restriction (PCR; reduced food intake without malnutrition) is known for extending longevity in multiple species¹⁸. PCR is also an established approach to positively impact metabolic fitness and resistance to multiple forms of acute stress¹⁹⁻²¹. Despite these wide-ranging benefits, potential clinical applications have never been considered feasible in humans due to the practical difficulties associated with voluntary food restriction combined with the assumption that PCR benefits take a long time to accrue. Recent preclinical studies by the applicants reveal a rapid onset of PCR benefits against surgically induced traumatic inflammation, IR injury, and the fibroproliferative vascular response to injury in rodents^{14-17, 22, 23}. Mechanistically, rapid changes in adipose phenotype and associated adipokine profiles appear to underlie these benefits¹⁶; but the key mediator appears to be endogenous H₂S. This field thus stands poised to move to clinical utility, and vascular surgery offers an optimal risk/benefit ratio for translation of the PCR/H₂S hypothesis¹⁵.

For the current project we propose a randomized, controlled trial to evaluate patient compliance and biologic mechanisms of a short-term pre-operative PCR diet in comparison to a normal ad libitum diet for 4 days before elective vascular surgery involving an open major operation. Both Endpoints relate to the long-term primary scientific objective to test the hypothesis that ***brief upregulation of endogenous H₂S via pre-operative PCR in elective major surgery improves clinical outcomes in humans***. After a successful pilot study of the PCR diet conducted inpatient before carotid endarterectomy titled Short-Term Endogenous Hydrogen Sulfide Upregulation (PHRC Protocol No. 2016P002507), we now aim to expand the study to at home diet among a variety of vascular surgery procedures. The primary endpoints of the study are:

- 1) Feasibility, and in particular outpatient subject compliance with PCR (measured by direct dietary intake data and serum markers including plasma free amino acids, pre-albumin, and insulin-like growth factor in comparison to the ad libitum diet).
- 2) Measurement of H₂S and standard biological markers of stress in blood collected at baseline, immediately before surgery, and after surgery including stress hormones, oxidative stress, and inflammation. We will also collect intra-operative biologic samples of discarded tissues and a gram of adipose tissue (peri-vascular, subcutaneous) to inform potential mechanistic links between PCR induced H₂S upregulation and any perturbations in peri-operative stress responses.

A secondary exploratory aim is to compare clinical outcomes and complications between groups. These endpoints will include surgical and medical complications such as cardiac, neurologic, infectious, vascular,

wound, and any other clinically significant events that occur within 30 days of surgery. We will also look broadly at one-year survival and major cardiovascular events, though it is acknowledged that the project lacks fiscal resources to fully power this mechanistic trial for clinical efficacy.

Research Strategy (Background, Significance, Preliminary Data, Approach and Methods)

New Approaches are Needed to Reduce the Morbidity and Mortality of Vascular Surgery Procedures Elective surgery presents an opportunity unique in medicine: the planned stressful event. The need for and submission to surgery represents a major disruption in most patients' family and professional lives (including morbidity and mortality). Surgical mortality rates vary up to 1000 fold^{24, 25}, with vascular surgery carrying one of the highest rates of perioperative events. Much effort has been expended in studying methods of assessing and modifying the risk of vascular surgery²⁶. Several factors continue to challenge care in these patients. First, the population undergoing vascular surgery is getting sicker (e.g. higher prevalence of octogenarians²⁷ and diabetics²⁸). Furthermore, appropriate medical therapy to reduce CV events, particularly β -adrenergic blocking agent administration, remains an issue of great contention²⁹. Thus, the aging population, rising incidence of the metabolic syndrome, increasing vascular surgery access, and the lack of agreed upon mitigating medical therapy creates an environment susceptible for increased CV events with associated morbidity and mortality, all at enormous economic cost.

Compounding these problems, there is now evidence that the rates of cardiac injury after vascular surgery are far higher than previously recognized with the focus shift of events from perioperative MI to myocardial injury. One in seven (129/782) vascular surgery patients had biomarker evidence of cardiac injury³⁰; 5% of vascular subjects had a MI with an associated five-fold increased risk of mortality in the first 30 days³¹.

Basic Mechanisms Underlying Adverse Events in Vascular Surgery Have Been Defined Abnormal activation of the stress response by surgery has been associated with adverse CV events. Three components of the stress response have been studied in relation to postoperative complications: the sympathetic nervous system, oxidative stress, and inflammation. Vascular surgery increases catecholamine release³²⁻³⁴; for instance norepinephrine and cortisol are increased postoperatively in these patients³⁵. While still evolving, some clinical trials have demonstrated that antagonism of this system (β -blockers) reduces the risk of perioperative CV events^{36, 37}.

Oxidative stress participates in the stress response^{38, 39}. Reactive Oxygen Species (ROS) are produced in the vascular wall in response to injury^{40, 41}. Patients with atherosclerosis undergoing vascular surgery manifest elevated levels of oxidative stress⁴². In addition to acting as signaling molecules and mediators of the stress response, ROS individually and in combination contribute to many abnormalities associated with vascular disease, including vascular endothelial dysfunction and attenuated bioavailability of nitric oxide (NO)⁴³. Diminished NO bioavailability has been associated with increased rates of cardiac injury during vascular surgery⁴⁴. Patients undergoing both aortic aneurysm repair⁴⁵ and bypass surgery⁴⁶ manifest increased levels of oxidative stress post-operatively, possibly through an ischemia-reperfusion mechanism.

Inflammation is now recognized as a causative mechanism underlying atherogenesis⁴⁷ and acute clinical events related to atherosclerosis⁴⁸⁻⁵¹. Elevated inflammation markers predict both first CV events⁵² and recurrent events⁵³. High sensitivity C reactive protein (hs-CRP) strongly predicts postoperative MI⁵⁴. Pre-operative hs-CRP elevation was associated with a 2.5 fold increased rate of postoperative events⁵⁵. Statins reduce markers of inflammation and reduce postoperative cardiac events by 45% in vascular surgery patients⁵⁶, potentially via H₂S. Thus any strategy that can modulate inflammation, oxidative stress and sympathetic tone might reduce adverse events after vascular surgery. We propose pre-operative PCR as such a strategy.

PCR Appears to Positively Impact the Acute Stress Response via H₂S Upregulation An emerging method of improving the response to stress is PCR^{15, 16}. Defined as reduced food intake without malnutrition, PCR is best known for extending lifespan in model organisms from yeast to non-human primates^{57, 58}. The efficacy of PCR against acute stress, including surgical stress, in preclinical models is also well established^{19, 59-64}. Pilot studies suggest that humans respond to PCR in beneficial ways with respect to metabolic fitness (including improved glucose homeostasis, lipid profiles, and CV performance)^{65, 66}. But humans also have great difficulty complying with long-term voluntary food restriction, which is a prime reason that PCR has not been previously exploited in the clinical setting despite promising efficacy. However, recent data in model organisms point to a rapid onset of PCR benefits and challenges the notion that long-term PCR is required for benefits to accrue. In fruit flies, the maximal benefit of PCR on mortality risk occurs within 2-3 days⁶⁷. In rodents, we have shown that surgical

stress resistance also occurs within days^{19, 68}, and the benefits center on upregulation of endogenous H₂S¹¹. Nonetheless, with the exception of pre-operative overnight fasting (which serves a different purpose⁶⁹) dietary recommendations are largely absent from peri-op management⁴.

Pilot data suggest that adverse post-operative outcomes linked to oxidative stress, inflammation and stress hormones may be modified by brief PCR in humans as well. In subjects who undertook food restriction for 12 hours per day for a month, markers of inflammation decreased significantly⁷⁰. PCR has been shown to reduce oxidative stress in both chronic⁷¹ and acute⁷² settings. PCR may decrease stress hormone release⁷³, and it also appears to work rapidly even in obese individuals or ill individuals. In gastric bypass surgery, two weeks of PCR reduced the risk of complications and procedure difficulty⁷⁴. With respect to patient compliance, brief (4 days to 2 weeks) pre-operative dietary interventions have been shown to be feasible and safe in selected patients, ranging from obese candidates for laparoscopic surgery⁷⁴ to living organ donors⁷⁵. Our own pilot work involving humans showed that the proposed PCR diet is feasible and without excess risk for CEA patients before surgery.

Pre-operative PCR Modulates Response to Ischemic Injury The root cause of both MI's and strokes is ischemia resulting in cell death. The artery is clamped during an open vascular surgery. In most cases, restoration of blood flow (e.g., with unclamping), or reperfusion, is beneficial for cell survival and return to organ function. However, the return of oxygen and nutrients comes at the cost of increased free radical generation by inappropriate activation of cellular oxidases such as xanthine oxidase, further increasing oxidative damage. Inflammatory mediators further exacerbating tissue injury. While it is well known that long-term PCR (three months or greater) protects against ischemia-reperfusion injury to multiple organs in preclinical models, we have recently shown that robust protection from renal and hepatic IR injury can be obtained within 3 days of total food deprivation, with beneficial effects on survival observed in as little as one day¹⁹. Damage can be mitigated without restriction of food intake *per se*. *Ad libitum* diet lacking total protein or the essential amino acid tryptophan can still provide protection against renal and hepatic IR injury⁶⁸. The conserved amino acid deprivation sensor Gcn2 is required for the benefits of tryptophan deficiency against IR injury, implicating this nutrient sensing pathway for the first time in protection from surgical stress⁶⁸. The other major amino acid sensing pathway in mammals is controlled by the signal transducing kinase mTOR. Inhibition of mTOR by rapamycin increases longevity in rodents, possibly by mimicking nutrient deprivation. Our data indicate that mTOR also plays a role in stress resistance mediated by PCR. Using a genetic model in which mTOR is constitutively activated in the liver by genetic deletion of the mTOR repressor Tsc1, we recently found that PCR no longer protects against IR injury. We also recently linked the biologically active gas H₂S to the protective effects of PCR¹¹.

Short-Term PCR Attenuates Intimal Hyperplasia Restenosis is a recurrent problem from vascular patients and applicable to a range of procedures. While fortunately an infrequent event, restenosis after CEA can lead to strokes and repeat interventions for patients⁷⁶. In a validated mouse model of the primary driver of restenosis, intimal hyperplasia⁷⁷, we discovered that a short-term pre-operative PCR diet resulted in less intimal area (p=0.032) vs controls. Mice on a PCR diet also had significantly larger internal elastic lamina length (p=0.003), a remodeling measure. Finally, the PCR cohort serum exhibited significant decreases in certain classes of circulating lipids, including a collapse of multiple triglyceride species, suggesting a fundamental shift in metabolism toward beta oxidation. In addition, the lowered levels of circulating triglycerides may help reduce secondary insulin resistance and inflammation.

Approach and Methods

Eighty subjects undergoing carotid artery endarterectomy, aortic aneurysm repair (open, and endovascular if groin cut down planned), open lower extremity arterial procedures (bypasses, aneurysm repair, arterial and bypass graft reconstructions), major amputation of the lower extremity (below knee and above knee amputations), or open hemodialysis procedures either symptomatic or asymptomatic disease will be recruited and enrolled at BWH. Using a randomized (3:2), parallel design, patients will receive either the PCR diet (n=48; ScandiShake [any of 4 flavors] mixed with almond milk, calculated individually for a total daily volume to achieve 30% caloric restriction and 70% protein restriction, based on body weight and activity level), or continued routine *ad libitum* diet (n=32). The Mifflin St. Jeor equation will be used to calculate the total 24-hour energy needs based on gender, age, height, weight, and activity factor for the PCR diet. Daily physical activity will be assessed by questionnaire to determine the activity factor for accurate calorie restriction calculations. The general ratio recipe for the ScandiShake is 85 grams ScandiShake powder to 240 cc almond milk. Water

intake is ad libitum for both cohorts, and both diets can be consumed throughout the day and night (except on the day of surgery). We have selected the ScandiShake as the PCR diet because it is currently the lowest protein shake available and there are other trials ongoing with this approach, though none [except our prior pilot study] are in vascular surgery patients. We paired the shake with almond milk since it is also low protein. Patients will consume their assigned diets for the four days leading up to surgery until midnight the day of surgery when both cohorts will be NPO.

Inclusion Criteria: Patients greater than 18 years old who present for one of the following elective procedures at BWH:

- Carotid artery endarterectomy
- Aortic/iliac aneurysm repair (open, and endovascular if groin cut down planned)
- Open lower extremity arterial procedures (bypasses, aneurysm repair, arterial and bypass graft reconstructions)
- Major amputation of the lower extremity (below knee and above knee amputations).
- Open hemodialysis access procedures

Exclusion Criteria: Patient intolerance or allergy to any of the ingredients in the PCR diet; Active infection; Pregnancy; Malnutrition, based on abnormally low serum albumin (lower than 3 g/dL); Uncontrolled diabetes (HgbA1c greater than 12%); Substance dependency that could interfere with protocol adherence and assent as determined by the PI; Active non-cutaneous cancer under treatment with chemotherapeutics or radiation; Emergency surgery; Active participation in any another interventional or randomized study; Participation in the current study within the past 30 days.

Recruitment

Subjects will be recruited through the BWH Vascular Surgery outpatient clinics and inpatients wards. Physician-Investigators will notify the study coordinators about candidates that fit inclusion/exclusion criteria. Study coordinators may discuss the study with potential subjects in detail and answer questions about the research being conducted. Patients will be introduced to the study in outpatient clinics or inpatient units and given ample time (>12 hours) to consider enrollment. Patients will be provided with the opportunity to discuss the trial with family or another physician if they wish to consent at a later time. Consent will only be obtained by a licensed Physician Investigator. All patients screened for this study will either have an established relationship with the physician-investigator or an established collaboration between a clinician known to them and the physician-investigator. Only with the collaborative clinician's approval may the investigator or study coordinators approach the patient about the research trial. Once the patient's permission has been obtained, screening tests and procedures will be performed to determine subject eligibility.

If the patient consents and remains eligible for the study, then the subject will then be randomized. This randomization will occur before the OR event to allow for resources preparation, baseline testing, and dietary intervention.

Foreseeable Risk and Discomforts

Patients randomized to the PCR diet may have a difficult time adhering to the PCR four-day diet, however our pilot data shows that it is feasible for vascular patients to adhere to the diet pre-operatively. We hope to alleviate this by introducing a variety of flavors that patients may choose from. Furthermore, by requiring the patient to log their meals with either the MealLogger app or paper record, we can closely monitor and control dietary intake while also allowing the patient to continue their normal lifestyle before surgery.

Patients randomized to the PCR diet may also experience hunger or discomfort higher than normal from limiting their diet for four days. We hope that with additional flavors some discomfort can be alleviated. If the patient feels too hungry or uncomfortable they may choose to withdraw from the study. Patients may also experience fatigue pre-operatively if randomized to the PCR diet. Based on our pilot data of PCR diet research in humans, patients were able to complete the diet safely and without excess risk.

Due to the high quantity of carbohydrates in the PCR diet, hypoglycemia will not be a major concern for subjects. However, at baseline we will screen diabetic patients for glucose control by questionnaire and HgbA1c testing. Those with very poor glucose control (HgbA1c >12%) will be excluded. Patients over the age of 65 on hypoglycemic agents (ie sulfonylureas) will hold their hypoglycemic agent medication for the four-day PCR diet intervention. Patients who have a baseline HgbA1c value less than 7% will decrease their insulin by

20%. Patients who report daily episodes of hypoglycemia will be reviewed by our study staff endocrinologist for glycemic control. Recommendations will be made to insulin dependent patients to check their blood glucose four times a day before meals and fasting while on the PCR diet. Our study staff endocrinologist will be available for patient consult in the event of PCR related hypoglycemia to change patients' insulin regimen.

We anticipate that there will be no significant adverse effects of the H2S upregulation and PCR diet but do expect a 20% dietary compliance failure rate. However, there may be additional risks or discomforts that are not known now.

Adipose biopsies add approximately 30 seconds to 1 minute to total surgery time but pose no other known risks.

Intellectual property and data generated under this project will be administered in accordance with Harvard policies. Materials and data will be transferred between BWH and material transfer and use agreements are already in place for such transfers. Publication of data shall occur during the project, consistent with normal scientific practices. Research data which documents/supports/validates research findings will be made available after the main findings have been accepted for publication.

Inadvertent disclosure of private medical information is a risk for study patients. Data records will be maintained in the locked Vascular Surgery Clinical Research office. Electronic files will be maintained on the Partners network drive, or securely input into selected professional data management system REDCap with access limited to study staff only.

Benefits

Patients may or may not benefit medically from taking part in this study. Theoretically patients receiving the PCR diet may have less chance of complications after surgery, but this is unproven. We do not expect to observe any physiological benefit if patients are randomized to the non-dietary restricted arm.

Data Collection

Time	< 30 Days Pre-op (Baseline)	4 days Pre-Op	3 days Pre-Op	2 days Pre-Op	1 day Pre-Op	Day of Op (Pre-Op)	Recovery Area	Post- Op Day #1	Post- Op Day #30 (+/- 14 days)	Post- Op 1 Year
Patient Screening (estimated n=600)	Enrollment, Randomization, Education (n=80)	Control Continue Usual Dietary Pattern (2:5 randomization)						NPO except meds	Resume Usual Diet	Study Participation Terminates
		Dietary Restriction Protein-Calorie Restriction x 4 days (3:5 randomization)						NPO except meds		
MealLogger app Entry		X	X	X	X	X	X		→ Post Op Day 14	
Blood Draw						X	X			
CBC with diff, Chem 7, lipidomics, cortisol, epinephrine, norepinephrine, dopamine, hs CRP, IL-6, leptin, HMW adiponectin, resistin, IGF-1, P3, serum pregnancy test (as needed); pre-albumin (baseline and preop)		X								
Determinations										
Physical Activity Questionnaire	X									
24-Hour Food Recall	X									
Height, Weight	X					X	X		X	
eGFR	X					X	X			
Wound Evaluation								X		
Tissue Collection*						X				
Full Medical History	X								X	
Satisfaction Questionnaire									X	
Physical Exam	X								X	
Chart Review										X
	Preprocedure					Inpatient		Post Procedure		Registry

*discarded artery, vein, peri-vascular or subcutaneous fat, aneurysmal specimens, and amputated tissues

Dietary compliance will be measured by way of dietitian calculated intake (for data analysis will be the gold standard). The subjects will be asked to document their meals using either the MealLogger app or paper

record. The MealLogger app allows subjects to take a picture of their meals, either the ScandiShake or their routine ad libitum diet. Study dieticians will interpret dietary intake through photos and directly contact subjects using the app for clarification of intake, or through paper record if subjects are unable to use the MealLogger app. Compliance with the dietary intervention will also be evaluated through measuring expected changes in the levels of plasma amino acids using an LC-MSMS platform, and insulin-like growth factor-1 (IGF-1) by ELISA. Because free amino acids are not stored in the body, PCR results in a rapid decline of essential amino acids, including methionine and tryptophan, that are monitored easily in blood plasma^{79, 80}. Isolated protein restriction also results in reduced plasma IGF-1, even in the absence of overall PCR⁸¹.

H_2S and H_2S production capacity (two distinct entities, see *Cell* paper¹¹) will be longitudinally measured, as will stress and safety markers in blood samples taken prior to dietary intervention (PCR or control), after the dietary intervention but before surgery, and sequentially after vascular surgery. Blood samples will also be collected and analyzed by Dr. Ho's team at Northwestern to assess for changes related to PCR. Adipose tissue biopsies from fat depots (subcutaneous, perivascular) will be taken at the time of surgery and analyzed for the effects of diet on lipid composition and on inflammatory markers predictive of the response to surgical stress. Additionally, discarded specimens (discarded artery, vein, peri-vascular or subcutaneous fat, aneurysmal specimens, and amputated tissues) will be collected and stored for microscopic analysis. Enrolled patients and their clinical data will be entered into a study-specific database within REDCap.

Statistical Considerations

The focus of this Mechanistic Trial is to gain insights into practical future trial construction considerations such as patient compliance and physiologic impact. We anticipate that the supervised PCR will be completed by a majority (~80%) randomized to that arm and that H_2S and H_2S production capacity will rise on day two and be significantly elevated above baseline until re-feeding POD#1. We expect that markers of stress will change significantly less in the active treatment group compared to normal diet group. Using an α of 0.05 and β of 0.8, based on the differentials observed in rodent work and factoring in non-compliance, via intent-to-treat the study is powered to test these two primary scientific markers (H_2S and norepinephrine/IL-6). Furthermore, we believe that there will be no significant adverse effects of the H_2S upregulation and PCR diet but do expect a 20% dietary compliance failure rate. Based on our own pilot work, we anticipate that markers of inflammation will be elevated (compared to non-atherosclerotic patient's samples in hand) in the enrolled subjects and further amplified by the surgical stress. We anticipate that the blood and adipose phenotypic signatures will have high inflammation, but that the subjects compliant with PCR will have a reduced global inflammatory activation state, and trends toward less carotid plaque inflammation and fewer postoperative complications. Our key endpoint for upregulation of endogenous hydrogen sulfide will be the P3 cell sorting assay. Based on pilot data we anticipate a 4% increase in the PCR diet cohort compared to ad lib diet controls, with an expected standard deviation of 8%. Using a power of 80% and an alpha of 0.05, and 8% specimen loss rate we need a sample size of $n=80$ to reach statistically meaningful conclusions (un-paired T-test). Importantly for future trials, we anticipate that the unbiased discovery platform will generate novel mechanistic hypotheses founded on human data and tissues that will be ripe for future research and translation.

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