Short-term pre-operative dietary restriction in vascular surgery

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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**).

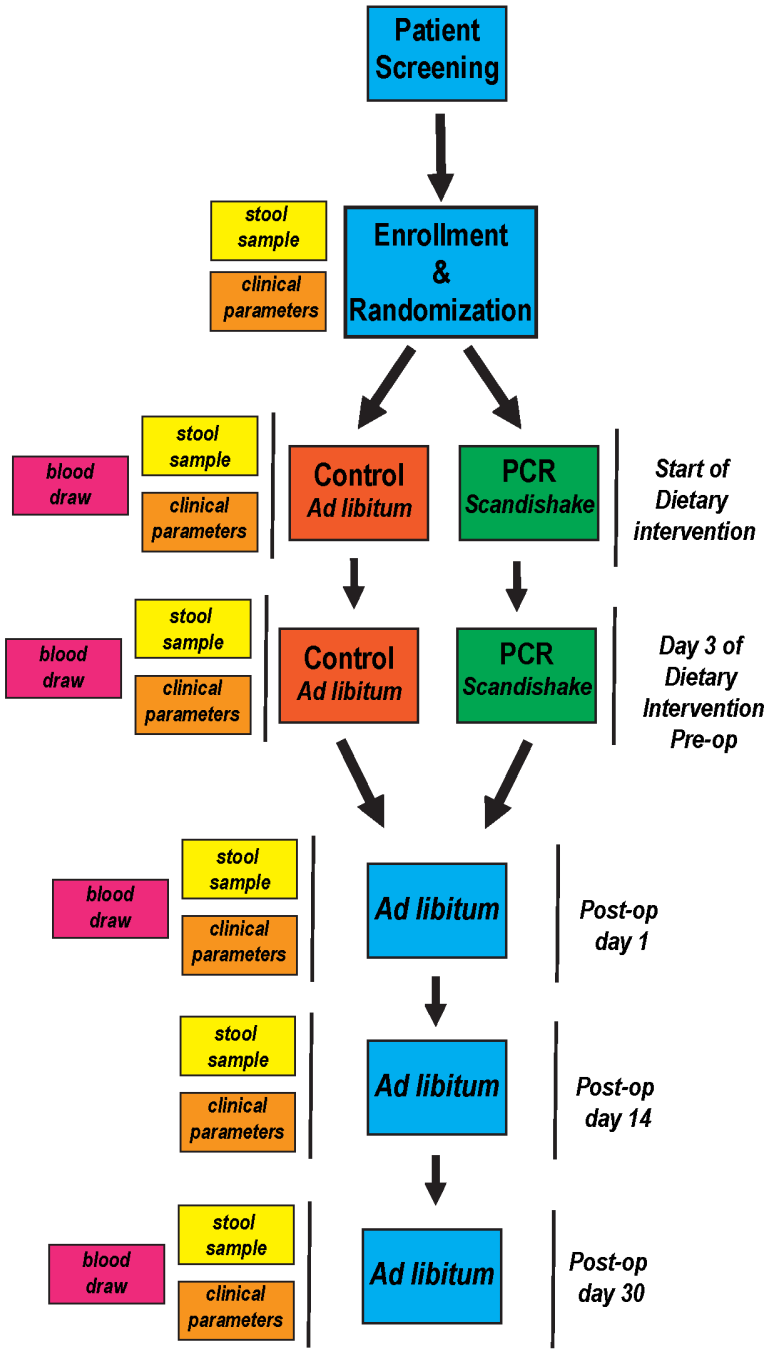

Figure 1. Study design

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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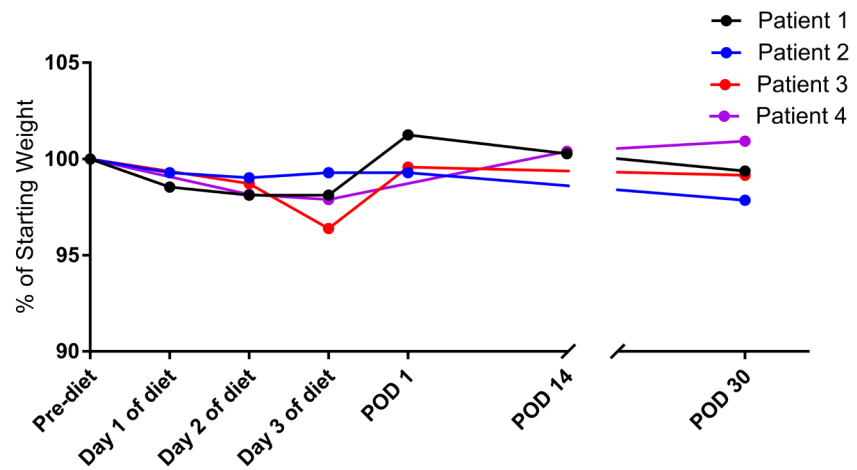

Figure S1. Weight loss during the study as percentage of starting weight. All patients remained within 5% of their starting weight.

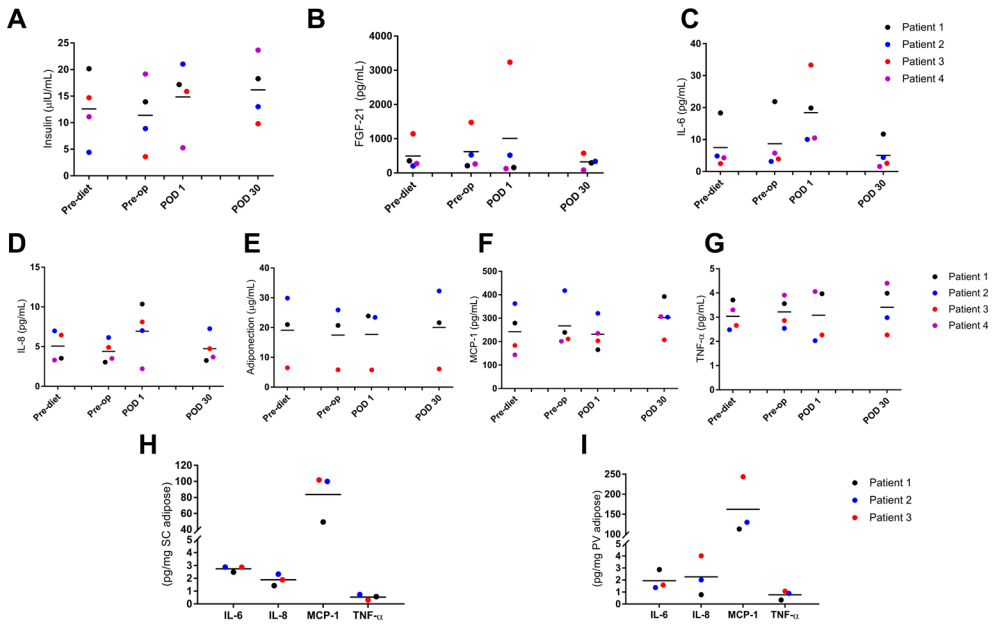

Figure S2. A: Insulin levels in μU/mL in all 4 patients during the course of the study, as measured by ELISA. **B:** Circulating FGF-21 levels in pg/mL during the course of the study, as measured by ELISA. **D-G**: Circulating levels of MCP-1, TNFα, IL-8 and adiponectin during the course of the study. **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.

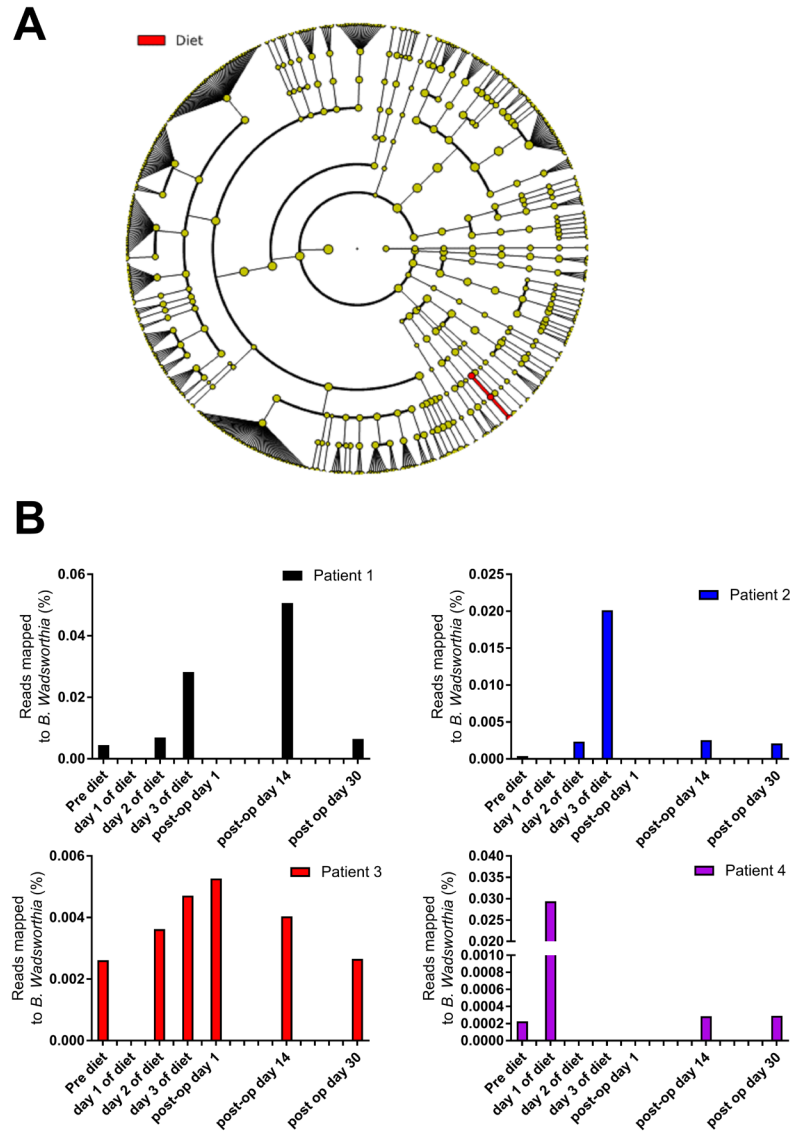

Figure S3. A: Differentially abundant bacterial taxa between the samples collected during the dietary intervention (n = 9) and the samples collected either before or after the dietary intervention (n = 11). Cladogram generated by LEfSe indicating differential relative abundance of *Bilophila wadsworthia*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

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