Short-term pre-operative dietary restriction in vascular surgery

Kip, P.

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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