Chapter 6

Summary, translational aspects and future perspectives, and concluding remarks
In this final chapter, the main findings of each research chapter are briefly summarised, followed by a more general discussion of translational aspects and future perspectives that focuses on testing novel therapeutic approaches against postoperative complications in mouse models. A detailed discussion of the research findings can be found in the respective research chapters.

1 Summary

Patients undergoing surgery often suffer from postoperative complications that are unrelated to the surgical procedure or surgical technique. Both the macro- and microvasculature of the cardiovascular system and various other organs can be affected, which can subsequently result in morbidity and mortality. Thereby, the benefit of surgery is consumed. Postoperative complications represent a growing global problem affecting between 5% and 70% of patients undergoing surgery, depending on various factors such as type of surgery performed and definition of postoperative complication used (1–3). Surgery-induced inflammatory processes have been identified as factors contributing to postoperative complications (as introduced in Chapter 1). A better understanding of these inflammatory processes in the macro- and microvasculature will help to rationally design therapeutic approaches to reduce perioperative complications. Therefore, advances in understanding and mitigating surgery-induced inflammatory processes involving the macro- and microvasculature are required in the context of perioperative complications.

1.1 Part 1 – Predisposed macrovasculature in the postoperative context

In Part 1 of this thesis, research involving the macrovasculature during and after surgery has been addressed. Atherosclerosis is a disease affecting the macrovasculature and it is the most common cause of cardiovascular disease (CVD). It is a common co-morbidity of patients scheduled for all kinds of surgery. Epidemiological studies have shown an inverse correlation of the extent of atherosclerosis and functional high-density lipoprotein (HDL) carrying cholesterol. Firstly, in Chapter 2, we studied the association of HDL cholesterol levels and HDL particle function with postoperative complications such as myocardial ischaemia in a patient cohort undergoing vascular surgery. HDL is known to be cardioprotective due to anti-oxidative and anti-inflammatory function (4). In addition, it plays a critical role in reverse cholesterol transport (revCT), a mechanism which is believed to reduce atherosclerotic burden by facilitating cholesterol efflux.
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from macrophages in the arterial wall. Lipoprotein analysis of plasma and HDL fraction revealed significantly reduced levels of HDL and LDL cholesterol. The extent to which HDL cholesterol decreased after surgery was highly significantly associated with the occurrence of postoperative myocardial ischaemia. Moreover, we have shown that revCT and antioxidative HDL capacity was reduced in human patient plasma samples 24 hours postoperatively compared to preoperative samples, however, an association to the occurrence of myocardial ischaemia was absent for revCT and only weak for HDL’s antioxidative capacity – contrary to what we expected. Altogether, these findings point towards a value of HDL as biomarker and therapeutic target to prevent macrovascular postoperative complications.

Following the line of research aiming to reduce the risk of postoperative atherosclerotic CVD, in Chapter 3, we assessed the role of the molecule regulator of G protein signalling 5 (RGS5) on atherosclerotic plaque development. RGS5 is a negative regulator of G-protein coupled receptors and is known to play a role in vascular development and arterial remodelling in vascular smooth muscle cells (VSMCs) and pericytes (5). Expression in other cells such as endothelial cells and macrophages have also been observed (6,7). Loss of RGS5 has been observed in established atherosclerotic plaques in mice and humans. The signalling pathways, which RGS5 is modulating, are currently not fully understood. In this thesis, I show that RGS5 deficiency significantly decreased plaque burden after 14 weeks of high fat diet feeding of double-deficient apoE RGS5 mice by reducing macrophage content and complexity of the plaque in an atherosclerotic disease model in mice. Based on our data, reducing RGS5 activity might be a therapeutic strategy to prevent macrovascular postoperative complications.

1.2 Part 2 – Microvasculature and the kidney in the postoperative context

Beyond the macrovasculature, capillaries in organs, the so called microvasculature, can contribute to the development of postoperative complications. We therefore tried to mimic surgery-induced changes in the microvasculature. As the kidney is an organ that is commonly affected by postoperative complications, Part 2 of this thesis centred around microvascular endothelial inflammatory activation in the kidney and microvascular targets to prevent postoperative complications. Surgery-induced cytokine release can lead to an exaggerated response of the immune system and subsequently to postoperative complications. Amongst others, tumour necrosis factor
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alpha (TNF-α) is an acutely elevated pro-inflammatory cytokine after surgery (8). It has been linked to systemic inflammation and endothelial dysfunction (9). Therapeutic strategies are required to dampen excessive pro-inflammatory signalling induced by TNF-α in various cell types of the vasculature. Patients undergoing coronary artery bypass graft (CABG) surgery with cardiopulmonary bypass (CPB) have an elevated risk of developing postoperative complications such as acute kidney injury (AKI) (10). CPB may trigger systemic inflammation due to contact of blood with the extracorporeal circuit, changes in endothelial shear stress, haemodilution, as well as organ/tissue ischaemia and reperfusion (11). In Chapter 4, we described an endothelial in vitro model incorporating plasma (20%) from patients undergoing CABG surgery with CPB and a change in flow rate (0 to 20 dyn/cm²) to mimic surgery-induced inflammation and changes in endothelial shear stress. Patient plasma collected before surgery, and again at 6 and 24 hours postoperatively, was used. We found elevated levels of the pro-inflammatory cytokine TNF-α in plasma, and plasma and urine markers of AKI. However, patients included were having a low preoperative risk of postoperative mortality and showed only mild signs of systemic inflammation and kidney damage. In our model, an increase in pro-inflammatory endothelial activation by expressing interleukin (IL-) 6, IL-8, E-selectin, intracellular adhesion molecule 1, or vascular cell adhesion molecule 1 was not observed at mRNA level following the exposure of endothelial cells to postoperative plasma and flow alterations. Our results did not confirm that microvascular pro-inflammatory activation by plasma components was underlying the observed postoperative increase in inflammatory and kidney injury markers in plasma and urine.

Although TNF-α signalling has extensively been studied, the signalling pathways and particularly the crosstalk in endothelial cells downstream of the TNF receptor 1 and 2 are not completely understood. The backbone of many signalling pathways are protein kinases that phosphorylate peptides in the presence of adenosine triphosphate in a reversible manner. Therefore, in Chapter 5, we studied the kinome profile of endothelial cells in culture following TNF-α exposure to find therapeutic targets for interference with TNF-α-induced pro-inflammatory signalling in microvascular endothelial cells. Using Pamgene’s flow-through arrays, phosphorylation of peptides was tracked and subsequently kinase activity was predicted. Kinome profiling revealed 64 protein tyrosine kinases (PTK) and 88 serine-threonine kinases that were differentially active in the TNF-α-induced endothelial response at different time points compared to
unstimulated control. The renal microvasculature consists of the four compartments arterioles, glomeruli, peritubular capillaries, and venules. We checked for the presence of a selection of PTKs in the different compartments by immunohistochemistry. We selected the PTKs AXL tyrosine kinase (Axl) and Fyn Src family tyrosine kinase (Fyn) for further studies. Pharmacological inhibition of either Axl or Fyn reduced pro-inflammatory responses in TNF-\(\alpha\)-stimulated endothelial cells at mRNA and protein level in vitro. Further, we studied leukocyte-endothelial cell adhesion interaction following the pharmacological inhibition of either Axl or Fyn, and we observed a minor reduction of leukocyte adhesion to endothelial cells in vitro. We concluded that kinome profiling can be a useful strategy to screen for kinases as therapeutic targets to attenuate TNF-\(\alpha\)-induced endothelial pro-inflammatory activation. Reflecting on this method for kinome profiling, the kinases are identified based on existing databases and thorough validation in the tissue of interest by conventional molecular and cell biology techniques is necessary to ensure biological relevance of the identified kinases.

In conclusion, our findings elucidate molecular mechanisms for therapeutic strategies to mediate pro-inflammatory activation in the macro- and microvasculature following major surgery. We show postoperatively reduced HDL cholesterol that correlates with the occurrence of myocardial ischaemia, indicating the potential of therapeutically modulating HDL and/or function in the operative context. We also demonstrate modulatory effects of RGS5 in the macrovasculature to reduce atherosclerosis in mice. In the microvasculature, we observed reduced endothelial pro-inflammatory activation upon pharmacological inhibition of Axl and Fyn in TNF-\(\alpha\)-induced pro-inflammatory activation in endothelial cells in vitro. For the purpose of novel clinical treatment options, further in vivo experiments are required to assess the therapeutic potential of the different molecules in the context of postoperative complications. Some of these research ideas will be discussed in the next section of this chapter.

2 Translational aspects and future perspectives

2.1 General use of mouse models to mimic patients undergoing surgery to study macro- and microvascular responses

Practical tools to improve postoperative patient care are biomarkers and therapeutic targets, for which animal models are indispensable methods for preclinical and translational research (12). Animal models allow for insight on multiple cell types, tissues,
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and organs, and provide information on cell-to-cell interactions. Albeit the translation of findings from animal models to human biology has limitations, preclinical animal models such as our surgical mouse model predisposed with atherosclerosis are useful and irreplaceable tools for studying pathological processes and testing interventions (13). In the following section, I outline the potential of a surgical atherosclerosis mouse model and how this model could be developed further to exploit its translational use in the context of postoperative complications.

2.2 Further application of the surgery mouse model in the atherosclerosis background

Rodents, and particularly mice, are the most utilised animal model, although mouse models have disadvantages such as differences in cardiovascular anatomy and physiology compared to humans and only 80% genetical overlap (14). Advantages such as easy genetic manipulation, low maintenance costs, short generation time, and ethical reasons compared to the use of other larger vertebrate animal models made mice the best choice as model for us (15). Since the early 1990s, different atherosclerosis models exist. At this moment, the apoE-deficient mouse model is, alongside the LDL-receptor KO mouse model, the most used atherosclerosis model. Albeit disadvantages and limitations of the apoE KO model, which have extensively been discussed elsewhere (13), the value of this model is widely recognised. Spontaneous lesions in the aortic sinus occur in apoE KO mice after three months of age, high fat diet feeding accelerates atherogenesis in apoE KO mice, and complex atherosclerotic plaques already develop after 7 weeks of high fat diet, as shown in Chapter 3. In combination with a surgical procedure, apoE KO mice represent a model of a pre-disposed organism, mimicking the common presence of co-morbidities in human surgical populations. Co-morbidities increase the risk of postoperative complications. Previously, a surgical model has been established (16) that mimics surgical stress by combining a laparotomy with a non-replaced 20% blood loss under general anaesthesia. Although effects on atherosclerotic plaque growth and complexity were observed, this model presents a milder version of surgery, as preliminary findings of inflammatory and kidney damage markers studied at mRNA level in kidney showed (data not shown). No persisting inflammatory response nor kidney damage was observed. Therefore, to study surgical effects in a mouse model predisposed with atherosclerosis, multiple modifications could be incorporated to establish a model with more severe effects. Firstly, haemorrhagic shock is one part of
the model, currently achieved by non-replaced 20% blood loss. However, studies have shown that loss of up to 60% retrobulbar blood loss are tolerated (17) albeit resuscitation with up to four times the lost volume after 1 to 3 hours was carried out, which were to be incorporated into the procedure. Other studies have evaluated repetitive retrobulbar blood sampling, which could be incorporated providing a presurgical baseline blood sample (18,19). For instance, one week prior to the surgical procedure combining laparotomy with blood loss, 15% of blood volume could be obtained via the retrobulbar plexus. Secondly, the existing surgical model could be advanced by incorporating longer durations of laparotomy under anaesthesia. Currently, the peritoneum is kept open for 30 minutes during laparotomy. Alternatively, escalating the degree of severity of surgery, cardiac surgery could be performed by adaption to an cardiopulmonary bypass model (20), with early signs of AKI, would be an option, although the technical set-up would require a complex heart-lung machine construction. Lastly, prolonging feeding durations of high fat diet to increase plaque size and complexity prior to surgical intervention would be possible to intensify the pre-surgical co-morbidity context.

2.3 Interventional studies in an advanced surgery mouse model with atherosclerosis

Endpoints of interest for me and my team were macrovascular atherosclerotic plaque growth and composition as well as microvascular endothelial dysfunction, and subsequent acute ischaemic events and AKI. With these different endpoints and clinical manifestations in mind, it would be interesting to perform interventional approaches such as HDL substitution, PTK inhibitors as well as RGS5 KO experiments in the atherosclerotic perioperative context to mimic the surgical patients with pre-existing CVD. HDL concentration and incidences of AKI have inversely been related after both cardiac and non-cardiac surgery (21–23). Assessing the effect of the surgical procedure with and without substitution of HDL on incidences of AKI in an advanced surgical mouse model, in which AKI markers are present and predisposed with atherosclerosis, would provide further insight into underlying pathological mechanisms. In the context of AKI, analysing the effect of pharmacological inhibition of the protein tyrosine kinases Axl and Fyn, identified in Chapter 5, on endothelial cell signalling in different surgical models would be of interest. Moreover, the effect of kinase inhibitors against i.e. Axl and Fyn is also interesting to be studied in the atherosclerosis context (24). Both Axl and Fyn have been shown to be potential targets in atherosclerosis. Especially for Fyn, a role in the infiltration as well as foam cell formation of macrophages has been suggested (25,26). Therefore, testing specific kinase inhibitors in the surgical atherosclerosis mouse
model and postoperatively analysing their effect on atherosclerosis development and incidences as well as degree of postoperative complications could be a potential future line of research.

Further advancement of the atherosclerotic mouse model could be achieved by incorporating a conditional KO of RGS5 to maintain physiological vascular development. As described before, RGS5 has been identified as marker for VSMCs and pericytes and a role in vascular development and remodelling has been shown by others (27). In Chapter 3, we have shown that pro-inflammatory macrophage content of atherosclerotic plaques was increased in global RGS5 KO mice. Assessing the role of RGS5 in different cell types in the atherosclerotic plaque is essential to determine the potential of this negative regulator of G-protein coupled receptors as therapeutic target. Further, the body of knowledge around other members of the RGS family has grown over the last decades and thus, other RGS molecules might also be of interest to be evaluated (28). With the clustered regularly interspaced short palindromic repeats and CRISPR-associated protein 9 (CRISPR-CAS9) tool, deficiencies of any specific RGS gene could be obtained, producing global or cell-specific, and traditional or conditional transgenic mice. Therefore, to understand the effect of RGS5 or other RGS molecules on signalling processes in atherosclerosis in the context of surgical stress, conditional RGS KO mouse models would provide a method to do so at the time of the surgical intervention.

Given that the conventional generation of atherosclerotic mouse models is costly and time-consuming, recent developments have shown that a novel atherosclerotic in vivo model can be generated with the use of the adeno-associated virus vector (AAV) encoding a gain-of-function mutant Proprotein-Convertase-Subtilisin/Kexin Typ 9 (PCSK9) (29). Following the generation of global and cell-specific conditional RGS KO mice by CRISPR-CAS9, these transgenic mice could be more easily transferred into the atherosclerosis background when applying the proposed AAV-PCSK9 approach. At this point (July 2023), we have generated a viable global conditional KO of RGS5 utilising a tamoxifen (TAM) inducible Cre-recombinase system. An VSMC-specific conditional RGS5 KO mouse has been generated by others (30). Generally, due to the heterogeneity in macrophage population and the overlap of markers with other myeloid cells, the development of macrophage-specific KO mice has taken a long time. Recently, a TAM-inducible Cre-recombinase system under control of the human CD68 promoter has been developed and thoroughly tested (31). Authors have validated this
model in the atherosclerotic context by injection with AAV-PCSK9 viral particles and subsequent high fat diet feeding for 16 weeks, followed by administration of tamoxifen and tissue collection. Cross-breeding this human CD68 promoter cassette driven conditional CreERT2 mouse model with a flippase recombinase system for RGS5 KO would provide a macrophage-specific conditional RGS5 KO model. Further, others have demonstrated that induction of atherosclerosis is feasible with a single injection of AAV-PCSK9 viral particles and subsequent high fat diet feeding (31). Using three different strains with different promoter-driven tamoxifen-inducible Cre-recombinase expression or with AAV-PCSK9 injection and high fat diet feeding will allow to assess the role of RGS5 in different cell types contributing to atherosclerosis. Combining these models with the surgical model, analysis of atherosclerotic plaque growth and complexity with cell-specific RGS5 KO will be possible. In summary, recent technical advances will make it possible to generate different transgenic mice strains quicker and cheaper than before, allowing us to research the role of RGS5 or other molecules in atherosclerosis, as well as the impact of therapeutic approaches targeting atherosclerosis on postoperative complications.

3 Concluding remarks
Postoperative complications occur in up to 70% of patients (1–3) and can lead to further morbidity or even mortality. Systemic and local inflammatory processes following surgery can cause postoperative complications and therapeutic strategies are required for prevention and treatment of postoperative complications. In this thesis, we identified underlying molecular mechanisms that may lead to therapeutic strategies to mediate pro-inflammatory activation in the macro- and microvasculature following major surgery as outlined in Figure 1. In Chapter 2, we showed that surgery induced a significant decrease in LDL and HDL cholesterol, the latter of which was strongly associated with the occurrence of myocardial ischemia in patients undergoing vascular surgery. In Chapter 3, we demonstrated reduced plaque growth and complexity upon RGS5 deficiency in apoE knock-out mice in vivo. In Chapter 4, we generated a model to study plasmatic components of patients during and after surgery and altered blood flow on endothelial inflammatory response. In Chapter 5, we identified the PTKs Axl and Fyn as therapeutic targets in microvascular endothelial inflammation induced by TNF-α.
Overall, we used preclinical in vitro and in vivo models combined with clinical data and biological samples to advance current understanding of macro- and microvascular
responses to major surgery to develop preventive strategies for postoperative complications. Future work could entail the advancement of an established surgical model to generate models with more severe complications. In the context of atherosclerosis, the novel AAV-PCSK9 murine model could be used to induce atherosclerosis, and traditional and conditional RGS molecule-deficient mice could be generated using CRISPR-CAS9. With these and other approaches we aim to reduce postoperative complications to diminish morbidity and mortality after surgery.
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Figure 1. Schematic outline of chapters studying macro- and microvascular responses to major surgery to develop preventatives for perioperative complications. Postoperative complications occur due to processes in the macro- and microvasculature. In Chapter 2, we showed an association between postoperatively reduced HDL cholesterol and incidences of myocardial ischaemia. In Chapter 3, we demonstrated reduced plaque growth and complexity upon Regulator of G-protein signalling 5 (RGS5) deficiency in apoE knock-out mice in vivo. In Chapter 4, we generated a model to study plasmatic components and altered blood flow on endothelial inflammatory response. In Chapter 5, we identified the PTKs Axl and Fyn as therapeutic targets in endothelial inflammation induced by Tumour necrosis factor alpha (TNF-α). Created with BioRender.com.
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Bibliography
