The role of immune regulating factors in the development of vein graft atherosclerosis = De rol van immuun regulerende factoren in de ontwikkeling van vein graft atherosclerose

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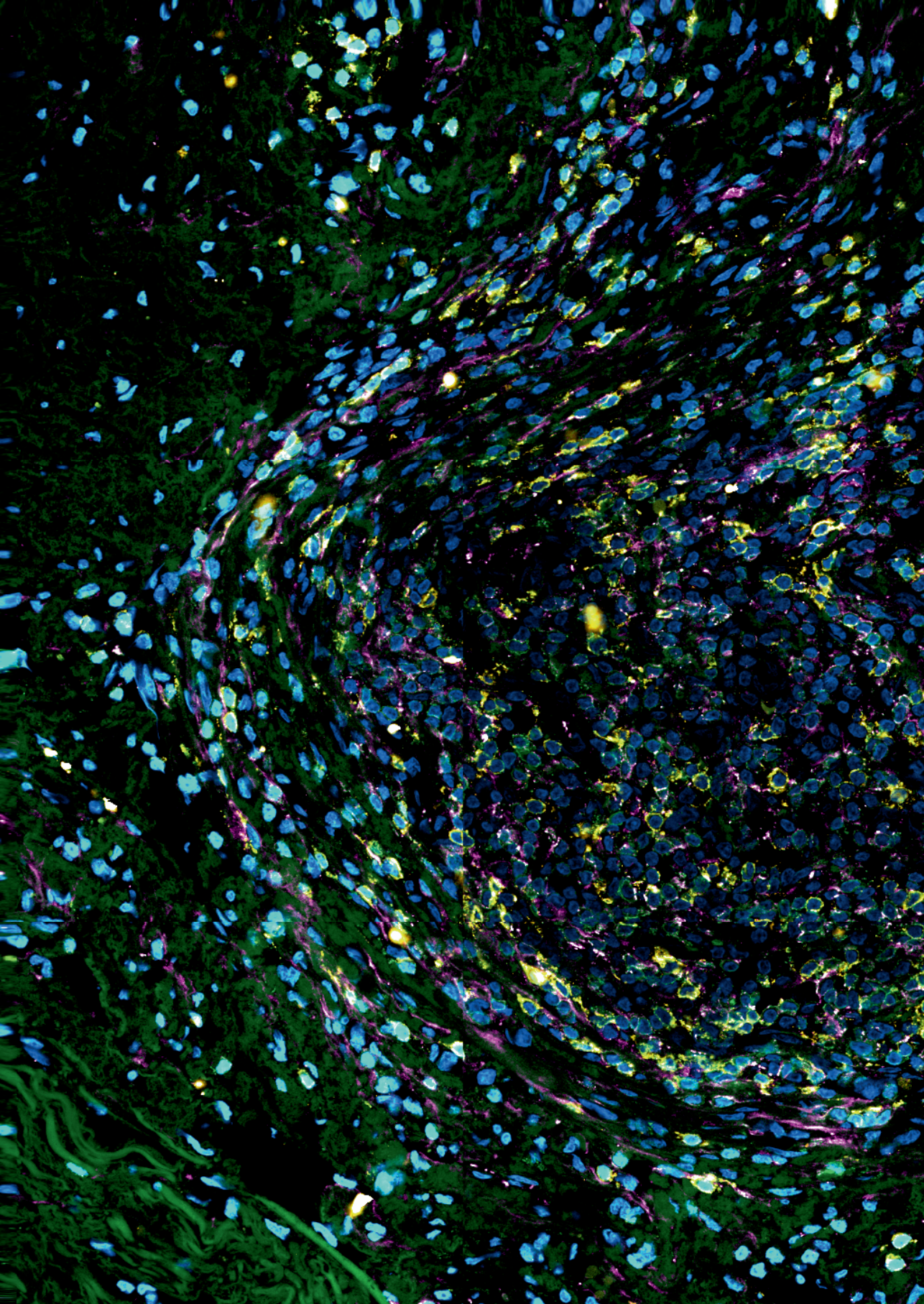

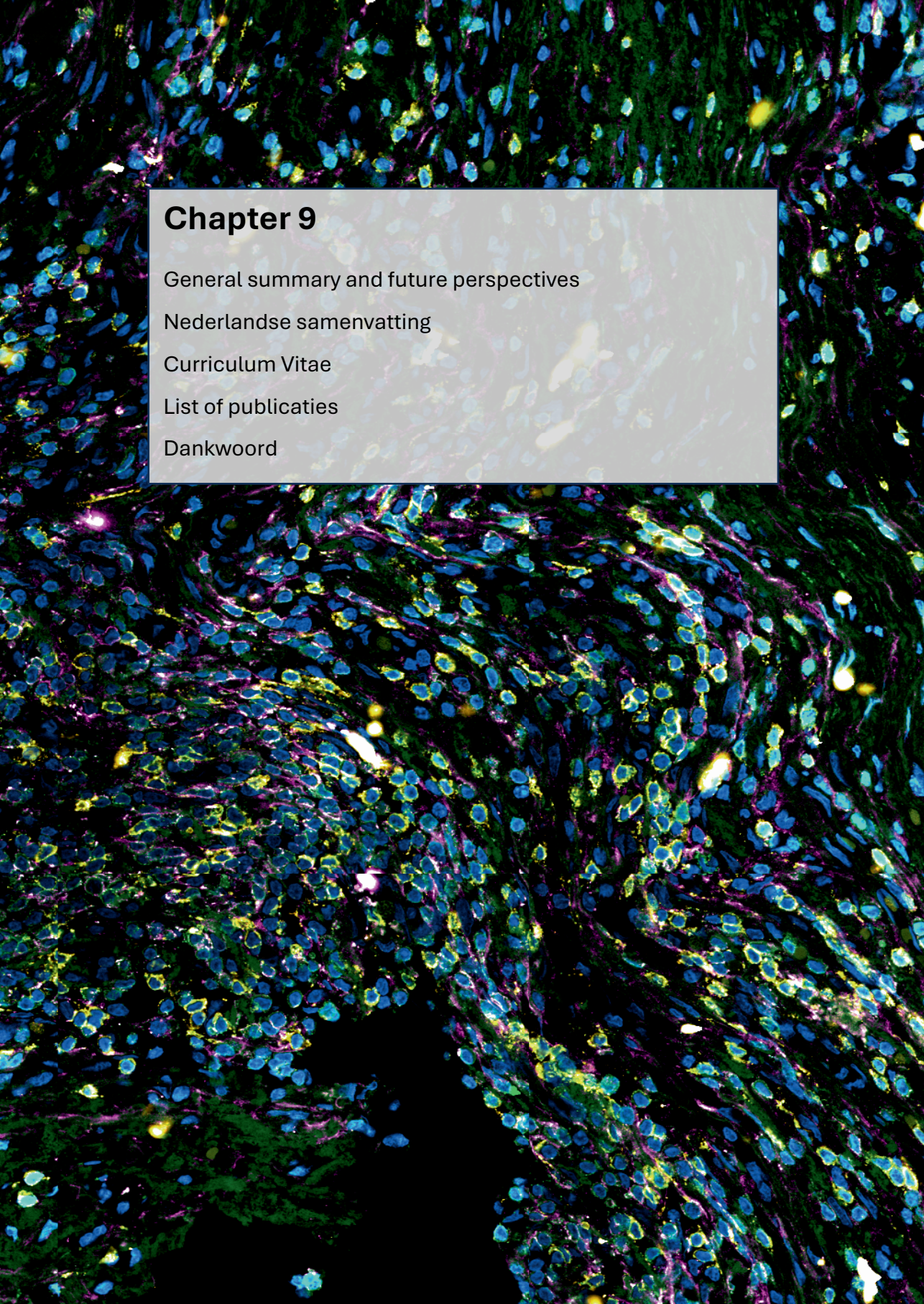

Chapter 9

General summary and future perspectives

Nederlandse samenvatting

Curriculum Vitae

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General summary and future perspectives

This thesis aimed to elucidate the role of immune regulating factors in atherosclerosis and accelerated atherosclerosis and exploit the therapeutical potential of targeting the immune system to enhance positive vascular remodeling. Atherosclerosis is a progressive disease characterized by the formation of lesions in the major arteries with accumulation of cholesterol, lipids, inflammation, calcification, and influx of immune cells. The size of the atherosclerotic lesion is the minor determinant, and the stability of the atherosclerotic lesion is the major determinant in clinical manifestations such as myocardial infarctions, cerebral strokes, or sudden cardiac death. Characteristics of unstable atherosclerotic lesions are a large necrotic core, a thin fibrous cap a high macrophage count, and T cell polarization towards the Th1 and Tc1 subsets. The human body can circumvent the stenosis via the maturation of arterioles and the sprouting of new capillaries to form new vasculature. T cells orchestrate this process by reducing the proliferation and migration of endothelial cells and enhancing the maturation to generate matured and functional neovessels. We found several immune regulating factors that can be the foundation for novel therapeutic strategies. Since T cells were shown to have a major effect on atherosclerosis and accelerated atherosclerosis, the first part of this thesis aimed to investigate the immune system involved in atherosclerotic lesion development and therapeutic options that can be targeted to reduce accelerated atherosclerosis. In the second part of this thesis, we focused on the imaging of vascular remodeling and compositional measurements with spectral photoacoustic imaging.

Several treatments to modulate T cell activation have been developed and successfully introduced as a cancer treatment. These immune checkpoint inhibitors block co-inhibitory pathways on T cells inducing T cell activation that results in tumor cell clearance. However, the role of co-stimulatory and co-inhibitory pathways are poorly described, and the outcomes of the use of

immune checkpoint inhibitors in CVD were unknown. In the review in **Chapter 2**, we provided an overview of the various immune checkpoint inhibitors in CVD. These immune checkpoint regulators can be either stimulatory or inhibitory and not only play a role in cancer but also in other diseases with immune modulatory aspects. We reviewed the role of co-stimulatory and co-inhibitory immune checkpoint inhibitors in CVD with special emphasis that treating cancer with immune checkpoint inhibitors can be lifesaving, however, also may result in cardiovascular toxicity. We provided a summary of the adverse events after immune checkpoint inhibitor treatment and underlined that reporting the type of adverse events is critical for future therapy selection. Subsequently, we argue that focusing on the inhibition of co-stimulation pathways in the future could be beneficial for the treatment of CVD.

The CD137 signaling pathway in VGD was investigated in **Chapter 3.** The T cell immune landscape of accelerated atherosclerotic vein graft lesions shows a progressive increase in the absolute number of CD4 and CD8 T cells, specifically Tem cells, as the atherosclerotic lesion develops. CD8 T cells, and to a lesser extent CD4 T cells, are activated during the early stages of vein graft atherosclerosis. We identified CD137 as a key costimulatory molecule locally expressed on CD8 T cells during this period. Targeting the CD137-CD137L signaling pathway with agonistic antibodies induced lesional T cell differentiation towards effector-memory cells and significantly increased the lumen area of the vein graft. In contrast, antagonistic CD137 treatment decreased the lumen area and promoted lesion growth. Notably, agonistic CD137 targeting improved lesion stability by impeding intraplaque angiogenesis and enhancing neovessel maturation, whereas antagonistic CD137 decreased neovessel maturation. The progressive increase in the absolute number of T cells in the atherosclerotic vein graft during lesion development is preceded by the rapid expression of CD69 and CD137, which are only expressed during the initial stages of lesion development. This expression indicates T cell activation

in the early stages of lesion development. This is further supported by the progressive increase in the number of Tem and Tcm over time in the vessel wall, particularly in CD8 T cells, which also exhibited higher CD137 expression compared to CD4 T cells. Treatment with agonistic CD137 antibodies enhanced the differentiation of both circulating and lesional T cells into Tem cells. This, along with the local expression of CD137 early after surgery, suggests a local effect on $CD137^+$ T cells in the vein graft that translates into a systemic effect. Additionally, agonistic CD137 treatment increased IFN-γ expression on lesional T cells, while systemic IFN-γ concentration also increased shortly after treatment. This was corroborated by a strong increase in IFN-γ production in vitro for both CD4 and CD8 T cells upon stimulation with CD137. The local upregulation of CD137 on T cells in the vessel wall during the early stages of lesion development renders this molecule an attractive target for early immunomodulation to improve vein graft remodeling and reduce atherosclerotic lesion development by inhibiting intraplaque angiogenesis.

Over the past decades, progress has been made in understanding the role of innate and adaptive immunity in vein graft failure (VGF), offering new opportunities to enhance prevention strategies. Chapter **4**, discusses the pathophysiological mechanisms underlying the development of VGF, emphasizing the role of immune response and associated factors related to VG remodeling and failure. Moreover, we described the effects of immune cells and related factors in early (thrombosis), intermediate (inward remodeling and intimal hyperplasia), and late (intimal hyperplasia and accelerated atherosclerosis) failure based on both preclinical (mouse) models and clinical data. Targeted immunomodulatory therapies could be developed to specifically address the different phases of vein graft failure. For example, therapies that modulate early inflammatory responses or enhance T cell activity might prevent thrombosis and intimal hyperplasia. Alternatively, we discussed the use of prosthetic materials as grafts. Tissue-engineered blood vessels present an

interesting alternative. We described various TEBV types that use scaffolds to attract or seed vascular precursor cells. TEBVs have potential as arterial bypass grafts and could be a future solution for vascular surgery, though they still need optimization before large-scale clinical application.

The contribution of the epigenetic factor PCAF in diet-induced atherosclerosis was studied in **Chapter 5**. The involvement of the epigenetic factor PCAF in vascular diseases has been suggested because polymorphisms in the PCAF gene were significantly associated with coronary heart disease mortality in elderly patients. Using the ApoE*3-Leiden PCAF deficient mice, we showed that PCAF deficiency increased atherosclerotic lesion sizes present in the aortic sinuses. It is known that PCAF is involved in the acetylation of histones at the site of NF-κB promotor genes. These pro-inflammatory genes like TNFα and IL-6 are involved in the progression of atherosclerotic lesion sizes. However, we were not able to measure differences in systemic TNFα or IL-6 levels in plasma between the control and PCAF-deficient mice. PCAF deficiency in mice is associated with a significant decrease in systemic FoxP3$^+$ Tregs resulting in an increase in atherosclerotic lesion size in the aortic sinus. Although atherosclerotic lesion formation is inflammation-driven and PCAF is associated with inflammation[12], we identified that PCAF deficiency in diet-induced hyperlipidemic ApoE*3-Leiden mice resulted in an increased atherosclerosis lesion size. We attribute this increase to a reduction in regulatory T cells upon PCAF deficiency since PCAF is also able to facilitate the activation state of the *Foxp3* gene and the FoxP3 protein, which is crucial in Treg differentiation. The mechanism of how PCAF can modulate T cell differentiation and subsequently influence atherosclerotic lesion progression needs to be identified. In conclusion, PCAF regulates atherosclerosis via the modulation of FoxP3$^+$ Treg differentiation.

In **chapter 6** we focused on the involvement of TGF-β signaling in the development of VGD. First, we examined the contribution of the ALK1 signaling pathway to vein graft remodeling in ALK1 heterozygous mice ($ALK1^{+/-}$) mice. Heterozygosity of ALK1 significantly increased the vein graft lumen area while not affecting the vein graft wall area. A significant increase in atherosclerotic lesion macrophages was found in the $ALK1^{+/-}$. Collagen and vascular smooth muscle cell content, two factors that indicate stability, were unaffected by ALK1 heterozygosity. Secondly, prevention of BMP9/10 to activate downstream signaling with an ALK1-Fc ligand trap resulted in an increase in outward remodeling in vein-grafted mice, both measured non-invasive with ultrasound and morphometric analysis. However, this positive effect of BMP9/10 ligand trapping was accompanied by an increase in vessel wall dissections, driven by destabilizing factors such as increased macrophage influx, decreased collagen content, and a reduction in vascular smooth muscle cells. Together, these results demonstrated an ambivalent role for ALK1 in vein grafts, characterized by macrophage-driven outward remodeling with the preserved luminal flow and an increase in destabilized lesions.

In the second part of this thesis, we examined the imaging capabilities to measure vascular remodeling and tissue composition with photoacoustic imaging. The capabilities to measure clinically relevant parameters for vascular remodeling in both VGD and arteriovenous fistula maturation were explored in **Chapter 7**. We quantified vein graft parameters including vein graft wall area and vein graft lumen area. With the high-fat high cholesterol diet as the intervention, we showed different vascular remodeling patterns in vein-grafted ApoE*3-Leiden compared to C57BL/6J mice. Over time, the 3D luminal volume was increased at 28 days relative to 3 days post-surgery. The vein graft wall volume was increased in ApoE*3-Leiden mice, with a constant wall size in C57BL/6J mice showing the capabilities of quantifying vascular remodeling. At the proximal side, the pulsatility index, resistive index, and peak systolic velocity

decreased longitudinally over time. Distally, the maximum acceleration increased by 56% in C57BL/6J VGs. Among the AVFs, 50% showed maturation after 7 days, based on a novel flow-criterium of 23 mL/min. Distinct flow patterns were observed at the anastomotic site and inflow artery of the AVFs relative to the control carotid arteries. Although this work is primarily observational with a description of methodological approaches, it serves as a platform for molecular analyses and therapy validation. To advance the imaging of vascular remodeling, **Chapter 8** focused on the method of using photoacoustic imaging to determine the vein graft tissue distribution and tissue changes over time. We investigated the performance of a newly developed unsupervised superpixel photoacoustic unmixing (SPAX) framework that enables fully automatic spectral unmixing and accurate quantification of molecular components. The SPAX approach has been first tested via a tissue-mimicking study and then validated *in vivo* murine model. Specifically, the *in vivo* longitudinal study included ApoE*3-Leiden and C57BL/6 mice. sPAI and SPAX data-driven analysis has enabled us to automatically identify oxy/deoxy hemoglobin components and lipid and collagen when present. Thus, the correlation between the unmixed spectra and the respective theoretical spectra has been evaluated longitudinally. This correlation was used as an indication to detect significant spectral changes related to mechanical variations during the inflammatory response. This study shows that sPAI enables unbiased compositional analysis of murine tissue. This approach offers valuable insights into vein graft dynamics and holds significant potential for advancing the non-invasive assessment of vascular diseases, ultimately supporting improved clinical decision-making.

Future perspectives

In this thesis, we reviewed different targets to reduce atherosclerosis and VGD. For this, we investigated the role of the adaptive immune system, innate immune system, and epigenetic regulators influencing the adaptive immune response. Significant advancements in cancer treatment have resulted in some improvement in the prognosis of cancer patients. However, the toxicity associated with these treatments can have adverse effects on the cardiovascular system, impacting both short-term and long-term outcomes[2]. To address this issue, several guidelines and articles have been published regarding the monitoring and management of cardiovascular health in patients undergoing treatment with immune checkpoint inhibitors[3]. Important is that the type of adverse event, cardiovascular disease risk, and malignancy should be recorded to calculate the risk of cardiotoxicity for a given patient. This risk of cardiotoxicity substantiates the discussion that short-term effects outweigh the long-term risks of coronary heart disease (CHD). From the vascular point of view, it would be interesting to determine if the vein grafts in patients receiving immune checkpoint therapy display enhanced remodeling. To study this, CABG or peripheral artery bypass graft remodeling can be measured in CHD patients who suffer from malignancies and receive immune checkpoint inhibitor (ICI) treatment. As stated in the introduction of this thesis, measuring vein graft stenosis in patients remains challenging but options are available as discussed below.

T cells play a role in VGD, and so T cell receptor profiling provides the most direct measurement of vein graft immunogenicity. A vein graft single-cell dataset may allow one to predict an objective preclinical response following CD137 therapy. Single-cell RNAseq experimentation provides the evenness, richness, and diversity of the T cell receptor[4]. These are used to describe the interactions with the environment and can be used to analyze the numbers of unique T cell receptors e.g. clonal expansion resulting in a value of clonal diversity. In VGD,

CD137 increases the activation threshold for T cells. Such co-stimulatory molecules may increase the T cell-mediated immune response. High clonal evenness may indicate an improved preclinical response to CD137[5]. A greater evenness means that there is an increased probability that a T cell will be able to recognize a vein graft antigen or activate via cytokines initiating an immunogenic T cell response. The hypothesis is that after CD137 treatment, the TCR distribution increases while the richness remains unaffected indicating that CD137 induces clonal expansion of T cells. However, the T antigens recognized by the more abundant TCRs are not known. To generate a new hypothesis on the influence of T cells in VGD and atherosclerosis, single-cell transcriptomics provides an opportunity to characterize cell-type-specific transcriptional networks, intercellular signaling pathways, and cellular diversity. In addition, this data can be used to reconstruct cellular networks, cluster cells of the same type, and link cell-type-specific transcriptional programs to vascular diseases[6]. This can lead to new immunological therapeutic targets, and validate the immunological shift associated with vascular remodeling.

Regulation of adaptive immunity by innate lymphoid cells (ILCs) is both directly via antigen presentation via MHC II and indirectly via the regulation of dendritic cells[7]. Innate lymphoid cells (ILCs) are a recently identified group of immune cells that play a crucial role in the body's early defense mechanisms and tissue homeostasis. Different subsets of ILCs, such as ILC1, ILC2, and ILC3, have distinct roles in modulating inflammation and tissue remodeling, processes that are central to atherosclerotic lesion development and progression[8-10]. However, dysregulation of these cells can contribute to atherosclerotic lesion instability and increase the risk of adverse cardiovascular events[11], highlighting their significance in the complex immune landscape of atherosclerosis. The complex triangle interactions between ILCs, T cells, and dendritic cells establish an interaction platform of both negative and positive feedback mechanisms, which has been studied in cardiovascular diseases[11].

However, due to the strong overlap with T cells, genetic approaches to deleting transcription factors do affect the T cell compartment resulting in confounding. ILCs isolated from atherosclerotic lesions and vein grafts compared to the more studied barrier ILCs can provide new tissue-specific therapeutic strategies. To note, most of the ILC knowledge is obtained from murine models limiting the translational meaning to the human cardiovascular disease setting.

ICI treatment such as PD-1/PD-L1 have revolutionized cancer therapy by modulating T cell responses[12, 13]. However, their role in CVD is complex, as they can exacerbate inflammation and lead to cardiovascular toxicity. Future studies could be directed to explore the dual effects of ICIs in vein grafted patients, focusing on identifying conditions under which they can be safely and effectively administered to modulate T cell responses. Targeting the immune system in combination with traditional lipid-lowering strategies may produce additive or synergistic effects, improving long-term outcomes.

In addition, combining immune checkpoint inhibitors that modulate T-cell responses with epigenetic modulators that suppress pro-inflammatory gene expression could be an effective strategy to both halt and reverse atherosclerotic lesion progression. Additionally, personalized medicine based on immune and epigenetic profiling will become critical in the selection of therapies. Advances in biomarker discovery could allow for the stratification of patients based on immune cell signatures and epigenetic markers[14], enabling therapies that address individual drivers of vascular disease.

An imaging platform could be developed to not only quantify morphometric parameters but also determine vein graft tissue composition and map its spatial distribution in a 3D rendering. This enables longitudinal tracking of location-specific compositional changes within vein grafts. In vein grafts, targeting an influx of immune cells could be the next therapeutical strategy to enhance vascular remodeling. Nowadays, the adhesion and migration of immune cells to

endothelial cells are described with super-resolution microscopy[15]. *In vivo*, tracking and quantification of immune cells. e.g. the diapedesis of T cells in vascular diseases remains undescribed. Photoacoustic imaging is capable of detecting single cells, based on a laser output of 532nm coupled to a 1000MHz transducer[16]. This setup could be used to validate if naïve T cells enter the vein graft, differentiate locally, and reside in the vein graft wall as effector memory T cells or whether the local vein graft T cells re-circulate in the peripheral circulation acting as a tertiary lymphoid organ. In the future, combining photoacoustic imaging with other advanced imaging modalities, such as super-resolution microscopy and molecular imaging, could further enhance the resolution and specificity of immune cell tracking. Additionally, the integration of machine learning algorithms could automate the analysis of imaging data, enabling the rapid identification of patterns and biomarkers associated with graft success or failure.

Artificial intelligence (AI) could revolutionize the treatment of atherosclerosis and VGD by accelerating both research and clinical applications. AI-driven machine learning algorithms can analyze vast datasets from genetic, epigenetic, and immune profiling studies to uncover novel disease regulators and identify specific therapeutic targets[17, 18]. In clinical practice, AI can enhance the precision of risk prediction models by integrating patient-specific data, including immune and epigenetic markers, to predict disease progression and generate intervention strategies based on big data. Furthermore, AI-assisted imaging techniques can improve the detection of plaque vulnerability and graft failure, enabling earlier and more effective treatments. As AI continues to evolve, its integration with immunomodulatory and epigenetic therapies may lead to highly personalized, dynamic treatment strategies, significantly improving patient outcomes with CVD.

In conclusion, this thesis demonstrated that immune-regulating factors, including epigenetic mechanisms and TGF-β signaling pathways, play a crucial role in the development and progression of atherosclerosis and accelerated atherosclerosis, offering potential targets for therapeutic interventions. Additionally, novel imaging techniques such as photoacoustic imaging have shown promising potential to monitor vascular remodeling and tissue changes, contributing to improved diagnostics and therapy validation.

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