Plaque stabilizing and destabilizing effects in atherosclerosis

Chapter 10

Impact

In this thesis, we investigated the role of three potential determinants of plaque destabilization: intraplaque microvessel presence and dysfunction, macrophage metabolism and fibroblast presence in atherosclerosis. In this impact chapter, we will describe the implications of the findings of this thesis for science and society and how obtained results were or will be disseminated to society.

The socio-economic burden of cardiometabolic diseases

Cardiovascular disease (CVD) ranks among the leading causes of mortality worldwide, with an estimated 17.8 million deaths in 2017[1]. It is the primary cause of death in Europe, in particular[2, 3]. Coronary artery disease and ischemic stroke, often caused by atherosclerosis, globally account for 50% and 15% of deaths accountable to CVD, respectively[1, 2]. Moreover, it has been predicted that if current risk factor trends persist, the number of CVD deaths will continue to rise[4]. The global burden of CVD is not limited to high mortality, as it is also associated with high morbidity and associated disability, hospitalization, and institutionalization[2, 5, 6]. Thereby, next to high social costs, CVD also comes with high economic costs. Indeed, CVD costs were estimated at €210 billion (direct and indirect costs) in 2015 and $320 billion (direct costs) in 2016 for the European Union and the United States, respectively[3, 5]. Current treatment options, specifically for atherosclerosis, include lifestyle modifications, lipid lowering drugs, antihypertensives, anticoagulants and ultimately, invasive surgery. Evidently, these treatment options do not sufficiently decrease CVD-related mortality and morbidity.

Next to CVD, we unexpectedly touched upon another cardiometabolic disease during my studies, namely metabolic dysfunction-associated fatty liver disease (MAFLD). MAFLD development is associated with an obesogenic environment, and disturbed lipid handling and glucose homeostasis[7]. MAFLD is the major cause of liver cirrhosis, hepatocellular carcinoma, and eventually, the necessity for liver transplantation. Globally, MAFLD prevalence is estimated at 25%[8], whereas annual direct medical costs approximate $103 billion in the United States and €35 billion in the Europe-4 countries (France, Germany, United Kingdom, Italy)[9]. It is associated with several comorbidities such as obesity, type 2 diabetes mellitus, hypertension, hypercholesterolemia, and even cardiovascular risk. Indeed, MAFLD is an independent risk factor for CVD. Additionally, associations of MAFLD with dyslipidemia, dysregulation of glucose homeostasis, endothelial dysfunction and systemic inflammation all contribute to increased CVD risk. Thus, tackling MAFLD is likely to reduce the risk of CVD and CVD-related mortality as well[10]. Currently, as there is no approved drug available, treatment of MAFLD is aimed at lifestyle modifications and treatment of underlying risk factors associated with MAFLD development and fibrosis progression.

Taken together, the aforementioned numbers clearly show that new treatment options are imperative to decrease the social and economic burden of both CVD and MAFLD.

The ripple effect of scientific research: stimulating scientific and societal advances in cardiometabolic diseases

The scientific impact of my thesis can be deduced from the influence of my findings on scientific advancements, acquisition of new insights, methods and theories, and the stimulation of future research. Moreover, societal impact is based on the potential contribution of my main findings to current societal challenges, such as the socio-economic burden of cardiometabolic diseases, in the short and long term. On the short term, the results of this thesis mainly contribute to novel scientific insights. We envision that these novel insights will stimulate further research on the short and long term, as they yielded new theories and tools and allowed us to identify several new potential targets for therapeutic strategies. Thereby, although this thesis did not directly yield therapeutic treatments for atherosclerosis and MAFLD, we expect societal impact in the long term, too. Below, I will shortly summarize novel scientific insights obtained per thesis chapter. Moreover, I will address how we expect our findings to stimulate future research and to contribute to therapeutic interventions and thus societal impact in the long term.

In **chapter 2**, we showed that the switch of cell-associated to soluble PDGF-B stabilized the atherosclerotic plaque. PDGF-B retention motif deletion also ameliorated diet-induced body weight gain and fat accumulation in liver and white adipose tissue. However, simultaneously, an immune response was stimulated as circulating immune cell levels were increased. Currently, it is rather unclear whether individual effects were conferred by deletion of the cell-associated PDGF-B isoform, or due to increased availability and secretion of the soluble PDGF-B isoform. Therefore, future research could focus on unravelling isoform-specific effects per PDGF-B-reactive cell type *in vitro* and in mouse models, to isolate beneficial effects and possibly harness those for future therapeutic interventions.

Although myeloid PFKFB3 inhibition did not affect atherosclerosis in **chapter 3**, we uncovered protective effects of myeloid PFKFB3 on MAFLD development in **chapter 4**. These results seem promising, but there are some hurdles to take in order to exploit this finding for therapeutic purposes in the long term. Firstly, relevance of our observations and protective effects of myeloid PFKFB3 on MAFLD should be confirmed in the human disease setting. The association between hepatic macrophage PFKFB3 expression and MAFLD development could be assessed in healthy and MAFLD human liver sections. Moreover, multi-lineage human liver organoids or co-culture experiments could be utilized to assess macrophage PFKFB3 expression in a fatty liver-enhancing milieu, such as (physiologically relevant) free fatty acid exposure, and to perform macrophage-specific knockdown or overexpression experiments[11]. For a therapeutic

treatment *in vivo*, it will be pivotal to target myeloid cells only, since in contrast to the protective effects of myeloid cell PFKFB3, hepatocyte PFKFB3 was associated with hepatic inflammation, macrophage recruitment and fibrogenesis in a liver injury model[12].
This could be accomplished using nanoparticles, carrying therapeutics that stimulate myeloid PFKFB3 levels, such as PFKFB3 mRNA or protein supplementation[13, 14]. Nanoparticles allow easy administration through intravenous injection and preferably accumulate in macrophages of the mononuclear phagocyte system (liver, spleen and lymph nodes), which could be used to our advantage[14, 15]. A recent review listed nanoparticle systems with preferential hepatic accumulation, and low to negligible accumulation in spleen and other organs[15]. Moreover, hepatic macrophage targeting could be further improved through addition of specific macrophage ligands to the nanoparticle, such as mannose[16]. Practically, it would be crucial to study the appropriate level of myeloid PFKFB3 overexpression or supplementation and confirm its protective effect on MAFLD development *in vitro* and in mouse models, and the absence of adverse side effects. In case of adverse side effects of PFKFB3 supplementation, it could be worthwhile to further explore the pathways that confer protective effects on MAFLD downstream of myeloid PFKFB3 inhibition, and target those. Lastly, our study only takes into account the effect of myeloid PFKFB3 inhibition from the start of fatty liver disease. MAFLD is usually only identified in patients in the clinic after progression to steatohepatitis or worse. Therefore, it will be essential to study whether myeloid PFKFB3 consistently confers protective effects throughout different stages of MAFLD and can induce regression of existing disease. This is required to establish suitable timepoints of treatment administration within the MAFLD disease process. Thereafter, the potential treatment would have to go through clinical trial phases. Taken together, we expect the entire track, from addressing remaining experimental questions until completion of clinical trials, to take another 10-15 years.

In **chapter 7**, we identified a pan plaque fibroblast marker which was associated with detrimental human plaque traits. This possibly indicates a harmful effect of fibroblasts in (certain stages of) atherosclerosis. Specifically, we also identified two fibroblast subclusters in the atherosclerotic plaque with divergent functions, related to regulation of angiogenesis and the inflammatory response. The identification of pan- and subset fibroblast markers is essential to increase understanding of fibroblast functions in disease, thereby driving scientific impact on the short term. Next to murine atherosclerosis, presence of the identified fibroblast subclusters and corresponding markers should be further validated in human atherosclerosis, using immunohistochemistry and scRNA-seq. Moreover, it will be crucial to further study the function of atherosclerotic fibroblasts in general, and of individual fibroblast subclusters, and their contribution to atherosclerosis. This could be achieved through fluorescence-activated cell sorting of fibroblasts from mouse and human plaques and subsequent *in vitro* investigation of functional characteristics and communication between fibroblasts and other cell types in atherosclerosis, such as macrophages. Alternatively, fibroblast (subcluster-

)specific ablation in mouse models could shine a light on the contribution of fibroblasts and individual subclusters to atherosclerosis development *in vivo*.
After further elucidation of fibroblast (subset) function in atherosclerosis, the identified pan- and subset markers will be pivotal for specific fibroblast targeting. Thereby, the findings of my thesis will drive scientific impact on the long term, and will potentially yield new leads for interventions. Indeed, specific targeting of detrimental or beneficial fibroblast subclusters could pose new avenues for therapeutic treatment. This could be accomplished using nanoparticles or extracellular vesicles, which is currently also under investigation for targeting tumor-associated fibroblasts[17, 18]. These methods would yield interesting opportunities for subcluster-specific removal of fibroblasts, for the modulation of expression of fibroblast-specific genes that are detrimental or protective in atherosclerosis, or to steer fibroblast differentiation through identification and regulation of involved transcription factors.

Lastly, next to cardiometabolic diseases, PFKFB3-expressing macrophages, PDGF-B-expressing and -reactive cells and fibroblasts can be found throughout the body in health and disease. Thereby, knowledge generated and markers identified in this thesis might benefit other scientific disciplines and stimulate or shape development of therapeutic treatments for other diseases. One rather specific example entails small molecule inhibitor PFK158, an inhibitor of PFKFB3, which has recently entered clinical trials for the treatment of solid tumor patients[19]. Although PFKFB3 selectivity of the inhibitor is under debate, our study in **chapter 4**, showing detrimental effects of myeloid PFKFB3 inhibition on the development of fatty liver disease, raises a call for caution in its usage.

Spreading the knowledge: dissemination of scientific research results
Dissemination of the research results obtained in my thesis is a prerequisite to ensure impact and return of investment, since research efforts are established through public funding. If results obtained in this thesis can lead to the development of therapeutic treatments for atherosclerosis and MAFLD in the long term, this will benefit a large patient population and their clinicians. Moreover, it is pivotal to transfer the results of this thesis to important potential stakeholders, such as the scientific community and pharmaceutical companies, but also to the public, to increase knowledge, stimulate awareness and collaborations, raise support and to accelerate the realization of therapeutic treatments. To reach the stakeholders, several chapters (**chapter 2, 3, 5 and 6**) of this thesis have been published in scientific journals, predominantly open-access. Combined, these articles were read more than 10,000 times, underscoring the stakeholder's interests in my investigations. The other chapters (**chapter 4** and **chapter 7**) are also in preparation for publication. Moreover, findings of this thesis were shared multiple times at national and international conferences (such as the International Vascular Biology Meeting in Oakland, California, USA), symposia and social media platforms such as Twitter and LinkedIn. Additionally, I was selected to give a Ted talk in

layman's terms at the Papendal vascular biology training course for PhD candidates, organized by the Dutch Heart Foundation, which allowed me to further develop my skills to present scientific research results to a broader, non-scientific audience. Further dissemination of our results to a non-scientific audience (cardiometabolic disease patients, other interested parties) will occur through giving lectures (e.g. via Hart & Vaat Café, https://www.hartenvaatonderzoekfondslimburg.nl/evenementen/hart-vaat-cafe, Harteraad, www.harteraad.nl) and lay summaries of my research, such as the one found in this thesis. Importantly, scRNA-seq data from **chapter 6** were deposited in the Gene Expression Omnibus (GSE196395). Moreover, these scRNA-seq data have also been made available for interrogation through PlaqView, an open-source single-cell portal for cardiovascular research (plaqview.uvadcos.io). This enables other researchers to examine the data with their own research questions in mind.

In conclusion, the data presented in this thesis provide insight into the role of microvessels and fibroblasts in atherosclerosis, and the role of macrophage metabolism in atherosclerosis and MAFLD. Although more in-depth studies are still required, we identified several opportunities for future research and therapeutic targeting in atherosclerosis and MAFLD.

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