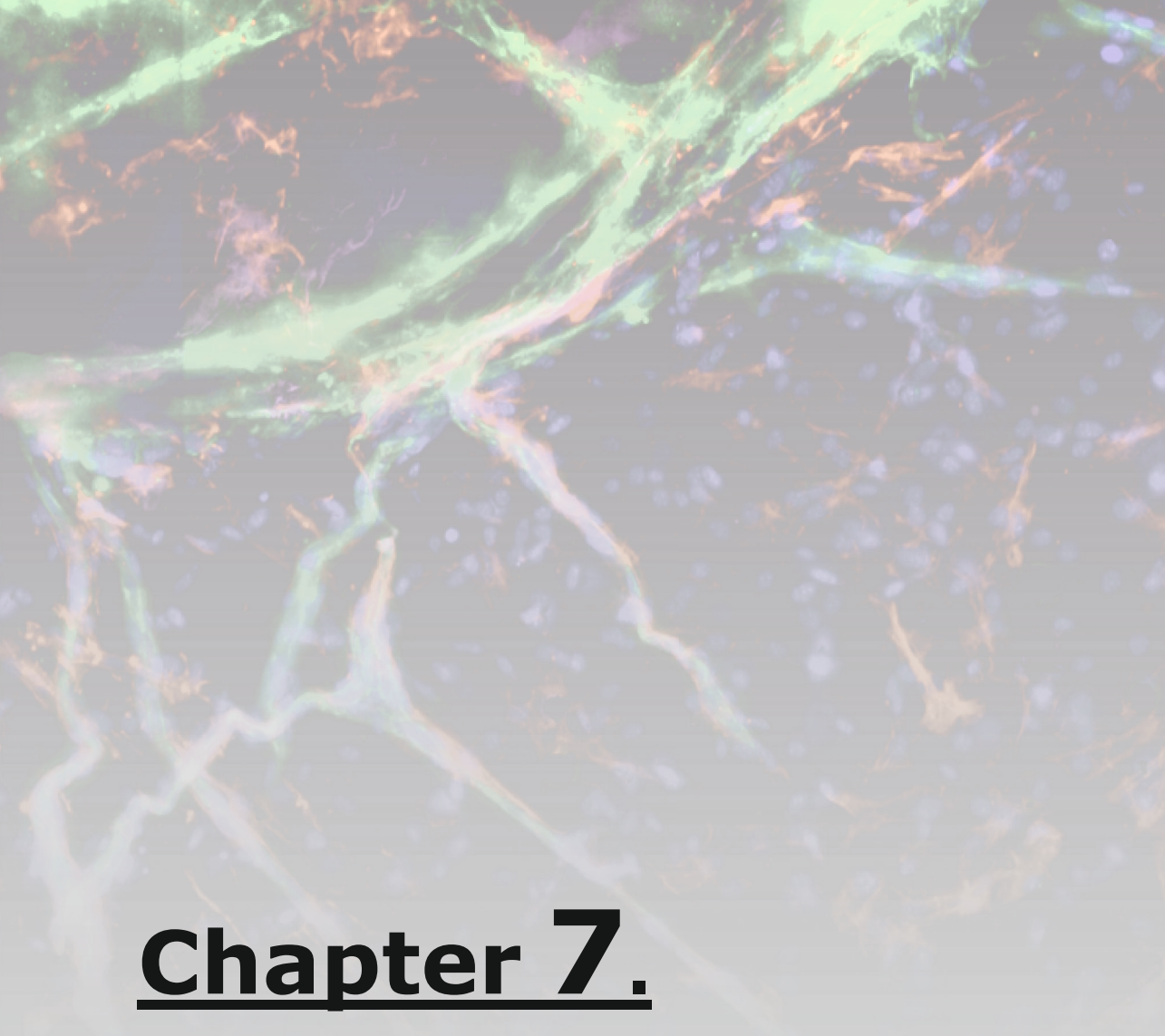
Targeting intraplaque angiogenesis : imaging and therapeutic interventions

Baganha Carreiras, F.



Chapter 7.

General Summary and Future Perspectives

GENERAL SUMMARY

Acute cardiovascular events, due to rupture or erosion of an atherosclerotic plaque, represent the major cause of morbidity and mortality in patients.[1] Substantial scientific evidences document a clear association between intraplaque angiogenesis and plaque progression toward an inflammatory and unstable plaque phenotype, leading to plaque rupture and ischemic clinical events.[2] Therefore in **chapter 2**, we discussed the pathological processes associated with angiogenesis in atherosclerotic plaques. We extensively reviewed the molecular mechanism behind vasa vasorum angiogenesis such as, hypoxia and EC metabolism. Moreover, we described the insights into neovessel immaturity and the relation with inflammatory mediators, and the subsequent effects of intraplaque hemorrhage, both in experimental models and in humans. We also highlighted the new therapeutic interventions and the prominent imaging modalities already in clinical studies to target plaque angiogenesis.

However, clinically available imaging techniques, such as PET, CT, and MRI do not have sufficient spatial resolution to visualize cellular events or image the detailed microvessels network in small size animal models.[3] Contrarily, two-photon intravital microscopy (2P-IVM) has emerged as a high-resolution approach for real-time evaluation of target dynamic processes, such as angiogenesis.[4] Therefore, **in chapter 3,** we describe how we used 2P-IVM to visualize and study the architecture of adventitial and intimal plaque neovessels in advanced atherosclerotic vein graft lesions in mice. Moreover, we hypothesized that quantification of the passive diffusion in healthy microvessels, continuous and fenestrated as well as diseased microvessels, might formulate a pattern to

evaluate microvessel leakiness of the plaque neovessels. To achieve that, we developed a 2P-IVM method to assess passive diffusion by quantification of 40 kDa TRICT-dextrans extravasation in real-time. We show that neovessels from ApoE3*Leiden mice vein graft lesions are pathological more permeable in comparison with healthy continuous and fenestrated microvessels. This 2P-IVM method is a promising approach to validate therapeutic angiogenic interventions targeting advance atherosclerosis in preclinical models.

Statins are the principal drug in primary and secondary prevention of coronary artery diseases due to their lipid lowering capacity. Interestingly, they can also, pleiotropically, provide additional benefits in the reduction of atherosclerosis. However, their effects on intraplaque angiogenesis and hemorrhage, major features in plaque instability, remain to be fully elucidated. Therefore, **in chapter 4**, we investigated the pleiotropic effects of atorvastatin in the ApoE3*Leiden mice vein graft model. In this study, we described atorvastatin dependent and independent lipid-lowering effects on accelerated atherosclerosis, including intraplaque angiogenesis and intraplaque hemorrhage. The ApoE3*Leiden mice were the ideal mice to perform this study since they respond to statins, as one of the few mouse models for atherosclerosis related research, but they also allowed the titration of plasma cholesterol levels by diet. Using this model, we could compare statin-mediated reduction of plasma cholesterol levels with diet-induced reduction and consequently, study lipid-lowering dependent and independent effects on plaque angiogenesis in the vein graft atherosclerosis lesions. Our findings show that atorvastatin modulates vein graft remodeling due to their lipid-lowering capacity and strikingly reduces intraplaque angiogenesis independently of their lipid-lowering effect. Moreover, atorvastatin pleiotropically improves

neovessels stabilization, leading to a reduction of intraplaque hemorrhage. Using a combination of *ex vivo* and *in vitro* assays, we confirmed our *in vivo* findings and we elucidated the pathophysiological molecular mechanism behind statins-mediated increase on capillary vessel maturation. We demonstrate that atorvastatin improves capillary vessel maturation by 1) inhibiting ANGPT2 release from ECs, 2) restoring Tie2-receptor activation and consequently, 3) preventing VE-Cadherin internalization and pericyte recruitment inhibition. Taken together, this chapter fully elucidates atorvastatin lipid-lowering dependent and independent effects on vein graft atherosclerosis, particularly on newly and hardly studied phenomenas, intraplaque angiogenesis and intraplaque hemorrhage.

Vein graft (VG) surgery is part of the standard revascularization strategies for patients with coronary and peripheral occlusive arterial disease. VG surgery can markedly improve survival and symptoms in selected patients.[5, 6] However, in ten years after surgery, only 60% of the VGs are still patent and improvements have been limited over the past decades.[7, 8] Therefore, **in chapter 5**, we extensively discuss the pathophysiological mechanisms underlying the development of VG failure, emphasizing the role of immune response and associated factors related to VG remodeling and failure. Moreover, we discussed potential therapeutic options that can improve patency based on data from both preclinical studies and the latest clinical trials.

Phosphocholine (PC) epitopes are modulators of the immunoinflammatory response and preventive anti-PC therapies have been developed to target VG failure. Passive immunization with anti-PC antibodies has shown to prevent VG atherosclerosis in a hypercholesterolemic murine model[9] by reducing inflammation and oxLDL uptake. Although intraplaque angiogenesis and

intraplaque hemorrhage play a major role in the additional supply of lipids and inflammatory mediators to the lesion, the potential role of anti-PC therapies on intraplaque angiogenesis and hemorrhage remained unexplored. Therefore, **in chapter 6**, we studied the effects of a humanized IgG antibody against PC (PCmAB) in advanced atherosclerotic lesions of the hypercholesterolemic ApoE3*Leiden mouse VG model. We found out that PCmAB stabilizes intraplaque angiogenesis by reducing EC proliferative and migratory behaviour and by improving neovessel integrity. PCmAB also seems to decrease intraplaque hemorrhage not only by decreasing the presence of erythrocytes in plaque but also by targeting M(Hb) macrophages *in vivo*. Interestingly, when we tested PCmAB effect on human cultured M(Hb) macrophages, PCmAB significantly decreased its CD163 expression and VEGFA secretion, suggesting that PC epitopes may also be involved in the CD163 scavenger activity. We also evaluated PCmAB effects on plaque morphometry and morphology. PCmAb decreased pathological intimal thickening and more importantly, increased lumen area, a clinical relevant parameter since it directly improves the blood flow. Additionally, PCmAB treated plaques have increased levels of collagen and decreased macrophage content and therefore present a more stable plaque morphology. Taken together, our findings reveal that PCmAB improves lesion stability, decreases inflammation and stabilizes vasa vasorum derived intraplaque angiogenesis. Therefore, PCmAB holds a promise as new therapeutic approach to target the vicious cycle between intraplaque angiogenesis, intraplaque haemorrhage and inflammation in advanced atherosclerotic lesions.

Regarding translation to the clinical setting, some considerations should be taken in account. It is known that preclinical animal models are not always predictive for efficacy of therapeutics in humans.[10] Although, the majority of genes involved in

disease processes are alike between most mammals, the availability and biological function of certain factors can differ between rodents and humans.[11] Therefore, the use of humanized animal models such as the ApoE3*Leiden mice, is, indispensable for good preclinical research. Additionally, attention should be given to treatment timespan and concentrations that can differ between animal models and humans. Interestingly, in the vein graft model, the starting point of disease is very clear: directly after the start of the operation.

FUTURE PERSPECTIVES

Intraplaque angiogenesis is associated with the initiation and progression of the atherosclerotic process and later implicated in plaque destabilization. Hypoxia, one of the factor driving plaque angiogenesis, is an hallmark in tumor angiogenesis and among the anti-angiogenic therapies that inhibit tumor angiogenesis some have been tested in atherosclerosis field.[3] However, in humans, the use of anti-angiogenic agents in clinical trials for cancer therapy shows that the anti-angiogenic drugs currently available increase the risk of cardiovascular events in atherosclerotic patients.[12] This is often related with beside compensatory responses given the extensive physiologically important functions of growth factors such as VEGF, it would be desirable to interfere with downstream players, such as the main orchestrators of vascular maturation. Indeed, established tumor antiangiogenic therapies that have also shown to normalization of the tumor vasculature by their reversal to a normal, stable and mature phenotype.[12] The decrease in microvessel density was accompanied by increased perivascular cell coverage. As a result, improved oxygenation of the tumour cells, inhibited of tumor growth.[13] Accordingly, improvement of integrity

plaque neovessel has been to decreased intraplaque hemorrhage and plaque instability as discussed in chapter 4 and others.[14, 15]

Since angiogenesis in atherosclerosis, is a dynamic process regulated by a large number of factors besides hypoxia, another logical step in development of new therapeutic opportunities would be targeting inflammatory factors promoting angiogenic-cell responses.[16-18] Guo et al. work on $CD163^{+}$ macrophages highlight an HIF1α-dependent angiogenic mechanism that relies on iron metabolism and CD163 expression rather than hypoxia.[19] Interestingly, PCmAB, as described in chapter 6, decrease intraplaque angiogenesis by improving neovessel integrity, and by targeting CD163 macrophage mediated VEGF release. Both, decreased intraplaque hemorrhage by targeting cell-specific and inflammatory-specific signaling events.

Therefore, future anti-angiogenic therapies should focus on restoring/maintaining microvessel integrity, which directly reduces intraplaque hemorrhage. Moreover, combination of anti-angiogenic and anti-inflammatory, can represent the future clinical regimens for long-term treatment of atherosclerosis, and prevention of plaque rupture.

Additionally, real time imaging of dynamic events such as intraplaque angiogenesis, is an exciting option to investigate temporal processes, such as the formation and maturation of plaque neovessels. Two photon intravital microscopy methodology, described in chapter 3, allows direct imaging of adventitial and intimal microvessels and more interestingly, quantification of microvessels permeability in a more realistic test environment compared post mortem tissue.

Finally, it can be concluded that the studies described in this thesis contribute to a better understanding pathophysiological mechanism underlying intraplaque

angiogenesis and intraplaque hemorrhage severity in atherosclerotic lesions. Moreover, this thesis explores new therapeutic strategies to improve plaque stability and new imaging options to visualize and validate vessel integrity-target therapies. Consequently, thesis definitely contributes to a better understanding of link between intraplaque angiogenesis, hemorrhage and inflammation.

REFERENCES

1. Hansson GK, Libby P, Tabas I. Inflammation and plaque vulnerability. J Intern Med 2015;**278**(5):483-93.

2. Michel JB, Virmani R, Arbustini E, Pasterkamp G. Intraplaque haemorrhages as the trigger of plaque vulnerability. Eur Heart J 2011;**32**(16):1977-85, 1985a, 1985b, 1985c.

3. Parma L, Baganha F, Quax PHA, de Vries MR. Plaque angiogenesis and intraplaque hemorrhage in atherosclerosis. Eur J Pharmacol 2017;**816**:107-115.

4. Taqueti VR, Jaffer FA. High-resolution molecular imaging via intravital microscopy: illuminating vascular biology in vivo. Integr Biol (Camb) 2013;**5**(2):278-90.

5. Garrett HE, Dennis EW, DeBakey ME. Aortocoronary Bypass With Saphenous Vein Graft: Seven-Year Follow-Up. JAMA 1973;**223**(7):792-794.

6. Favaloro RG. Saphenous vein graft in the surgical treatment of coronary artery disease. Operative technique. J Thorac Cardiovasc Surg 1969;**58**(2):178-85.

7. Yusuf S, Zucker D, Peduzzi P, Fisher LD, Takaro T, Kennedy JW, Davis K, Killip T, Passamani E, Norris R, et al. Effect of coronary artery bypass graft surgery on survival: overview of 10-year results from randomised trials by the Coronary Artery Bypass Graft Surgery Trialists Collaboration. Lancet 1994;**344**(8922):563-70.

8. Davis KB, Chaitman B, Ryan T, Bittner V, Kennedy JW. Comparison of 15-year survival for men and women after initial medical or surgical treatment for coronary artery disease: a CASS registry study. Coronary Artery Surgery Study. J Am Coll Cardiol 1995;**25**(5):1000-9.

9. Faria-Neto JR, Chyu KY, Li X, Dimayuga PC, Ferreira C, Yano J, Cercek B, Shah PK. Passive immunization with monoclonal IgM antibodies against phosphorylcholine reduces accelerated vein graft atherosclerosis in apolipoprotein E-null mice. Atherosclerosis 2006;**189**(1):83-90.

10. von Scheidt M, Zhao Y, Kurt Z, Pan C, Zeng L, Yang X, Schunkert H, Lusis AJ. Applications and Limitations of Mouse Models for Understanding Human Atherosclerosis. Cell Metab 2017;**25**(2):248-261.

11. Perlman RL. Mouse models of human disease: An evolutionary perspective. Evolution, medicine, and public health 2016;**2016**(1):170-176.

12. Carmeliet P, Jain RK. Molecular mechanisms and clinical applications of angiogenesis. Nature 2011;**473**(7347):298-307.

13. Goel S, Duda DG, Xu L, Munn LL, Boucher Y, Fukumura D, Jain RK. Normalization of the vasculature for treatment of cancer and other diseases. Physiological reviews 2011;**91**(3):1071-1121.

14. de Vries MR, Parma L, Peters HAB, Schepers A, Hamming JF, Jukema JW, Goumans M, Guo L, Finn AV, Virmani R, Ozaki CK, Quax PHA. Blockade of vascular endothelial growth factor receptor 2 inhibits intraplaque haemorrhage by normalization of plaque neovessels. J Intern Med 2019;**285**(1):59-74.

15. Michel JB, Virmani R, Arbustini E, Pasterkamp G. Intraplaque haemorrhages as the trigger of plaque vulnerability. Eur Heart J 2011;**32**(16).

16. Guo L, Harari E, Virmani R, Finn AV. Linking Hemorrhage, Angiogenesis, Macrophages, and Iron Metabolism in Atherosclerotic Vascular Diseases. Arterioscler Thromb Vasc Biol 2017;**37**(4):e33-e39.

17. Camaré C, Pucelle M, Nègre-Salvayre A, Salvayre R. Angiogenesis in the atherosclerotic plaque. Redox biology 2017;**12**:18-34.

18. Eltzschig HK, Carmeliet P. Hypoxia and inflammation. The New England journal of medicine 2011;**364**(7):656-665.

19. Guo L, Akahori H, Harari E, Smith SL, Polavarapu R, Karmali V, Otsuka F, Gannon RL, Braumann RE, Dickinson MH, Gupta A, Jenkins AL, Lipinski MJ, Kim J, Chhour P, de Vries PS, Jinnouchi H, Kutys R, Mori H, Kutyna MD, Torii S, Sakamoto A, Choi CU, Cheng Q, Grove ML, Sawan MA, Zhang Y, Cao Y, Kolodgie FD, Cormode DP, Arking DE, Boerwinkle E, Morrison AC, Erdmann J, Sotoodehnia N, Virmani R, Finn AV. CD163+ macrophages promote angiogenesis and vascular permeability accompanied by inflammation in atherosclerosis. J Clin Invest 2018;**128**(3):1106-1124.