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The natural history of human atherosclerosis : a histopathological approach

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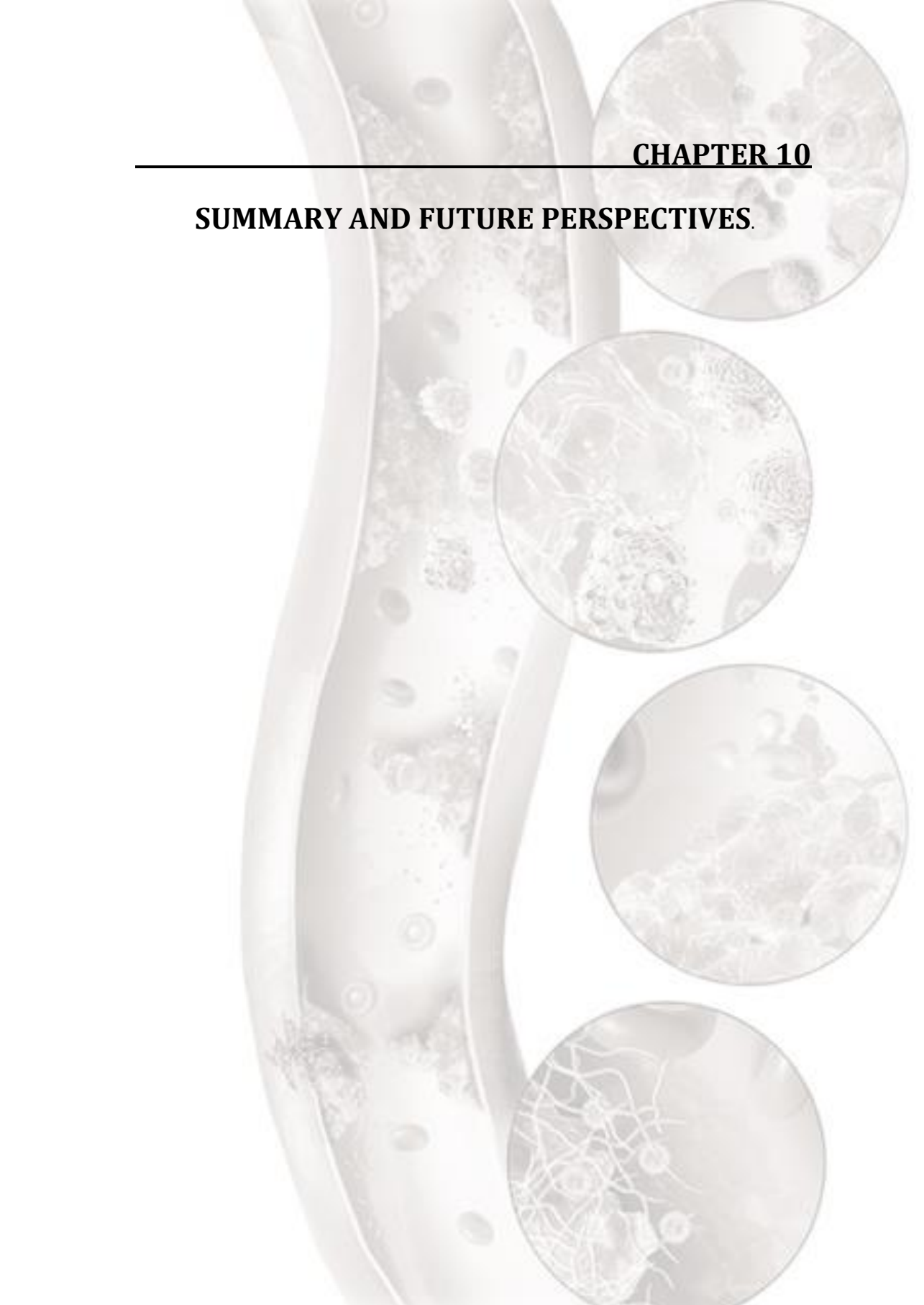


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CHAPTER 10

SUMMARY AND FUTURE PERSPECTIVES.

Summary

Atherosclerosis is the leading cause of death worldwide¹. A wealth of preclinical research performed over the past decades has led to many compelling hypotheses about the biological, pathophysiological and immunological bases of atherosclerotic lesion formation, and its complications such as myocardial infarction and stroke². Despite this progress, the leap from experimental animal findings to human atherosclerosis and clinical application presents many challenges^{3,4}. Essential gaps remain in translating experimental findings on how processes such as lipoprotein oxidation, inflammation and immunity contribute to human atherosclerotic disease and its complications. Bridging these gaps is required to achieve the full promise of scientific advances in atherosclerosis².

In an attempt to bridge the existing gaps we systematically evaluated the course of the human atherosclerotic process using a unique biobank containing over 500 individual peri-renal abdominal aortic wall patches, that were obtained during liver, kidney or pancreas transplantation, and over 600 coronary artery segments of the left coronary artery that were collected from healthy human hearts retrieved from Dutch post-mortem donors within 24 hours after circulatory stop and brought to the National Heart Valve for heart valve donation. These biobanks allowed us to use tissue from a group of apparently healthy individuals with an equal age and sex distribution, thereby avoiding potential bias introduced by the use of autopsy material from coronary death victims (mostly either young or old patients) or by the use of material from patients undergoing vascular surgery (generally end-stage atherosclerotic disease). Altogether the studies in this thesis systematically describe the critical morphological, pathophysiological and immunological characteristics of the human atherosclerotic process from initial non-progressive lesions to advanced unstable culprit lesions that underlie the clinical manifestations of human atherosclerosis.

Morphological aspects of the atherosclerotic process

Qualitative characterization of the atherosclerotic process fully relies on morphological classification based on histological plaque characteristics. Based on their observations from coronary death victims, Virmani *et al.* refined the AHA consensus classification in order to better reflect the heterogeneity of the advanced stages of atherosclerotic disease^{5,6}. It is unclear whether and how this human classification system based on the coronary artery can be applied to other vascular beds. In **Chapter 2** we describe and validate the coronary artery-based Virmani classification for the human peri-renal aorta. Aortic atherosclerotic lesions follow a similar pattern of progression as seen in coronary tissue, but grow far

beyond the size of coronary atherosclerotic lesions. Macrophages and foam cells play a large, contributing role in the evolution of the necrotic core, plaque progression and destabilization in aortic atherosclerosis. It is generally assumed that macrophages, through release of matrix metalloproteinases directly contribute to destabilization. The association between thinning of the fibrous cap and macrophage infiltration, and a significant decrease in macrophage content in healing ruptures underpins this assumption.

Carotid intimal media thickness (IMT) and coronary calcium scores emerged as epidemiological measures of atherosclerotic burden⁷. Yet, these measures are also progressively advocated as individual risk prediction tools, and changes in carotid IMT are now used as surrogate endpoints in clinical trials aimed at stabilizing or reversing the atherosclerotic disease⁸. **Chapter 3** tests the validity of IMT as a surrogate of the degree of atherosclerosis.

Results confirm an association between tissue IMT and plaque progression, but also show plateauing and even reductions in tissue IMT readings in the vulnerable and particularly stabilized lesions. Findings also show a large variability in tissue IMT, thereby resulting in wide confidence intervals around the trend.

As for calcium scores, calcium deposits reflect the later stages of the disease, and are particularly prominent in the so called stabilized lesions. Calcium scores should be considered a retrospective marker, identifying patients with manifest atherosclerotic disease, whereas a negative calcium score does not rule out presence of atherosclerotic disease.

Remarkably, while the majority of data on disease initiation and progression is based on information from mouse models of atherosclerosis, an unequivocal descriptive classification for murine atherosclerosis is however missing. In **Chapter 4** we show that the established “Virmani Classification” for coronary atherosclerosis can be universally applied for aortic and murine atherosclerosis, allowing a one to one comparison of murine and human atherosclerosis and a means of positioning preclinical findings in the human context.

The early stages of atherosclerosis in mice are comparable to the early stages of atherosclerosis as seen in their human counterpart. Based on the histological characteristics, it is concluded that the lesions in mice do not progress beyond an early fibroatheroma, and consequently that aspects of advanced and vulnerable lesion formation, and successive healing are missing in these models.

In direct comparison with their human counterparts we clearly see that the advanced lesions in mice are significantly smaller in size and lack an adventitial vaso vasorum. Due to the relatively small lesions in mice, an ischemic trigger is missing since the entire atherosclerotic process can still be sustained through

diffusion. Consequently, critical aspects such as ischemia and neovascularization are missing.

Molecular and cellular aspects of human atherosclerosis

A primordial event in atherogenesis is LDL accumulation in intimal layer of the vessel wall⁹. Extensive experimental data defines the role for oxidized LDL cholesterol in both progression and regression of atherosclerosis. **Chapter 5** systematically describes the relationship between the various oxidation-specific LDL epitopes (OSE), such as malondialdehyde-lysine (MDA2), E06, and IK17, and lipoprotein (a) (Lp(a)) in early and clinically relevant advanced human atherosclerotic plaques.

As lesions progress they become progressively enriched in apo(a), oxidized phospholipid (OxPL), and MDA related epitopes (IK17). Specifically the apo(a) epitopes were present in most of the early and advanced lesions, whereas OxPL, and IK17 epitopes were highly prevalent in foamy macrophages in thin fibrous caps, necrotic cores, and ruptured plaques suggesting that they are more closely associated with advancing and unstable plaques. The observed differences in immunoreactivity patterns presented among the different antibodies provide a framework for understanding the relationship between OSE and lipoproteins and the progression and destabilization of human atherosclerotic lesions. Specific epitopes are generated and/or enriched during different pathophysiological stages of lesion development, progression, and destabilization.

A crucial role for the cellular components of the innate immune system (in particular macrophages) is well accepted^{10,11}. It is now recognized that macrophages constitute a highly heterogeneous and dynamic cell population. Experimental studies mainly involving mice imply a major shift in macrophage identity during the atherosclerotic process with pro-inflammatory processes (classically activated M1 macrophages) dominating the initiation and progression phases of the disease, and alternatively activated M2 macrophages being the dominant phenotype in plaque resolution and repair¹².

Cellular components of the innate immune system are discussed in **Chapter 6** and were systematically evaluated throughout the process of human atherosclerosis, particularly in relation to complicated plaques, relevant to symptomatic disease.

A notable finding was that transition to pathological intimal thickening, the earliest form of progressive and irreversible atherosclerosis, is characterized by the accumulation of apolipoprotein B100 within the medial wall. This transition is

associated with a loss of internal elastic lamina integrity hallmarks the beginning of medial and adventitial involvement in atherosclerosis, a phenomenon that warrants further investigation.

Macrophages play a critical role in innate and acquired immunity. They are involved in all phases of atherosclerotic lesion development particularly in regards to lipid core formation, lesion remodeling, and degradation of the fibrous cap through release of matrix metalloproteinases, as seen in advanced symptomatic disease. Contrary to murine atherosclerosis, we found no evidence of a macrophage subclass shift during initiation, progression, rupture and ultimate stabilization of human atherosclerotic lesions. More interestingly, we identified a dominant class of macrophages that are double positive for both M1 and M2 markers challenging the paradigm of a clear M1-M2 dichotomy. Macrophage heterogeneity is far more complex than the current paradigm predicated on murine data and supports the involvement of additional (poorly-defined) macrophage subtypes or perhaps a greater dynamic range of macrophage plasticity.

Results show a strong positive relationship between progressive disease and fascin positive dendritic cells in all vascular layers. However, different roles have been identified for dendritic cells in atherogenesis and/or plaque progression in mouse models while a clear attribution to dendritic cells and the exact molecular mechanisms engaged remain unresolved.

Findings in human atherosclerotic lesions challenge claims based on murine models of the disease. Contrary to mice, mast cells are not associated with progression of human atherosclerotic lesions. Natural killer cells, abundantly present

in mice, are minimally present in human lesions. Murine studies imply a role for neutrophils in the initiation of the atherosclerotic process and plaque angiogenesis in later stages of the disease; our observational data in humans does not support a primary role of neutrophils. Neutrophil presence appears principally related to surgical manipulation of the aorta during removal.

Clearly, this divergence in observational data between murine models of atherosclerosis from our human atherosclerosis studies points to a limited translational aspect of animal findings.

The cellular components of the adaptive immune response are discussed in **Chapter 7** and were systematically evaluated throughout the process of atherosclerotic lesion formation, progression, destabilization and stabilization. Findings point to profound changes in the nature of the response in the lead up to plaque destabilization and show extinguishing of inflammatory processes upon plaque stabilization.

Fundamental differences exist between the human atherosclerotic disease and mouse models of the disease; particularly with respect to a very limited presence of regulatory T cells, absence of Th17 cells throughout the atherosclerotic process, and lack of B-cells in the early-, intermediate-, and final stages of the process. The earlier phases are dominated by diffuse cytotoxic T cell infiltration but progressive quantities of T helper cells are found during progression of the disease. Unlike findings in mice, we did not confirm Th1 dominance in human atherosclerotic process.

A remarkable and novel observation is the change in the inflammatory footprint that accompanies plaque destabilization and that fully resolves during plaque stabilization. On the cellular level this change is characterized by a sharp increase in the number of T-helper cells, emergence of naïve T-cells, and the appearance of B-cells and occasional plasma cells in tertiary follicle-like structures. CXCL-13 expression (a pivotal homeostatic signal for follicle formation) was exclusively observed in association with the follicle-like structures in the vulnerable lesions. Note; CXCL-13 was not detected in the other stages of the disease.

Our findings of a very restricted presence of B-cells in human atherosclerosis and gross absence of signs of B cell maturation in the infiltrating B cells exclude an autocrine or paracrine role of B-cells in the human atherosclerotic process. All in all, this study shows clear changes in the cellular components of adaptive immune system in anticipation of and during plaque destabilization. It is tempting to speculate that delineation of this chain of events may provide clues for early culprit lesion detection and plaque stabilization.

On the basis of animal studies, the AP-1 pro-inflammatory pathway has been implicated in the initiation and progression of atherosclerotic disease^{13,14}. As such AP-1 signaling has been brought forward as a critical correlate in the initiation and progression of vascular dysfunction and atherogenesis, and AP-1 inhibition has been proposed as an attractive target to prevent progression of atherosclerosis. In **Chapter 8** we demonstrate that in the early and later stages of human atherosclerosis, active AP-1 is indeed present in the aortic wall. In order to test for a possible role of AP-1 activation in the perpetuation of advanced atherosclerosis we quenched AP-1 activation by administering doxycycline to patients with peripheral vascular disease in a double-blind cross-over study. The absence of an effect of doxycycline administration on all activation markers investigated led to the conclusion that although AP-1 (and thus AP-1 inhibition) may be involved in the development of early atherosclerosis, it has no significant role in the later, clinically apparent stages of atherosclerosis.

Acute manifestations of atherosclerosis are thought to essentially reflect destabilization and rupture of a pre-existing vulnerable thin cap fibroatheroma⁵. Because of their comprehensive effects on inflammation and matrix homeostasis, members of the Transforming Growth Factor-Beta (TGF- β) super family of ligands (TGF- β , Activin and Bone Morphogenetic Proteins (BMP)) have been proposed as critical regulators of the atherosclerotic process in a large number of animal studies^{15,16}. The role of TGF- β and BMP signaling pathways throughout the complete spectrum of human atherosclerosis is evaluated In **Chapter 9**. TGF- β family members exhibit comprehensive anti-inflammatory effects on a wide range of cells, including endothelial cells, macrophages and T-cells, as well as fibroblasts and smooth muscle cells. Effects are all mediated by the classical signalling Smad phosphorylation pathways. Our results show that TGF- β family members affect all vascular associated cell types, in all three layers of the arterial wall throughout all stages of human atherosclerosis. Progression of atherosclerotic lesions into more complicated plaques (*viz.* progressive atherosclerotic lesions, vulnerable lesions and stabilized lesions) is not paralleled by changes in Smad phosphorylation.

All in all, our findings do not specifically characterize TGF- β and BMP signaling as key regulators in the progression and complications of atherosclerosis.

FUTURE PERSPECTIVES

Work in this thesis shows remarkable morphological parallels between disease progression in commonly used atherosclerotic mouse models and the human pathology. Despite these clear similarities, gross differences exist between advanced atherosclerosis in mice and men. In particular all aspects associated with cap maturation and plaque destabilization are missing in the murine models. These essential gaps hinder the translation of experimental findings to the clinical context in which plaque destabilization is thought to underlie all acute manifestations of atherosclerotic disease. Addressing the translational difficulties and gaps is a major challenge for future atherosclerosis research. Future research would benefit from the development of a more authentic human-like model; one that particularly incorporates aspects of the cap biology such as fibrosis and neovascularization in advanced lesions.

In this respect there have been several promising advances in porcine models of atherosclerosis that could prove essential for exploring the processes in late atherosclerosis¹⁷. Pig models have a more human-like size cardiovascular anatomy and are of closer physiologic and genetic resemblance to humans than rodents. Progressive atherosclerotic lesions in pigs exhibit overall morphology and several specific histopathological features that are shared with human lesions but not seen

in mice and include plaque neovascularization, intraplaque haemorrhages and solid calcifications similar to different stages of calcification in human lesions¹⁸. It is however important to note that also in porcine models, thrombotic complications due to atherosclerosis and advanced calcified plaques are rare, showing that even in this model critical aspects of human atherosclerosis are missing.

A remarkable observation in our vascular biobank material is the dominance of fibrotic calcified plaques (FCP) in middle aged individuals. According to the Virmani classification FCP represent the end-stage of atherosclerotic disease with plaque consolidation following rupture and healing^{5,6}. The high prevalence of (multiple) FCPs samples in our biobank implies that all these patients experienced plaque rupture(s) with subsequent healing without any apparent clinical symptoms. On a morphological level the majority of FCP are almost entirely acellular and contain condensed calcified remnants of the necrotic core. On this basis referring to these lesions as a fibrotic calcified scar (FCS) rather than a calcified plaque (FCP) seems more appropriate.

The studies in this thesis confirm the postulated differences in inflammatory involvement in human and murine atherosclerosis¹⁹. Whilst NK cells, regulatory T cells and Th17 cells are established factors in murine atherosclerosis, they are virtually absent in human atherosclerosis challenging a role for these cells in human atherosclerosis. Our data on human atherosclerosis also point to a considerable macrophage heterogeneity and diversity that is far more complex than the simple dichotomous classification scheme as proposed for macrophage differentiation in mice. Standard histological methods fall short to explore this variability. Although technically challenging, single-cell analyses could provide invaluable insights into further studying macrophage heterogeneity.

Atherosclerosis in mice critically depends on an immunological background of Th1-cells²⁰. Cytotoxic T-cells and T-memory cells are continuously abundantly present in human atherosclerosis whereas naïve T-cells and B-cells are almost exclusively present in the vulnerable phase. The absence of B-cell maturation suggests that this solely reflects a change in homing signals rather than an antigen driven response. B-cells are considered critical players in the atherosclerotic process; albeit their role, being either protective or detrimental, is still under debate and requires special attention in future research.

In rodents anti-inflammatory treatment almost indiscriminately results in plaque regression. Although anti-inflammatory treatment is widely considered a valid target in humans, it is important to point out that this optimism is not supported by clinical data. Potent anti-inflammatory actions have been firmly established for statins and ACE-inhibitors on the vascular wall²¹. However, this does not translate

in a clinical superiority to other forms of cholesterol respectively blood pressure lowering treatment^{22, 23, 24}. One might argue that the pleiotropic anti-inflammatory effects of statins and ACE-inhibitors are too weak to achieve the desired clinical effect. In this context it is important to note that systemic inhibition of IL-1 β also failed to show a positive effect on the function and structure of the arterial wall in aortic and carotid atherosclerosis²⁵. This challenges the commonly assumed canonical role of inflammation in atherosclerosis, and might suggest that the inflammatory component in atherosclerosis is merely an epiphenomenon rather than causative. This last notion is further supported by our interventional study where quenching AP-1 activation had no significant role in the later, clinically apparent stages of atherosclerosis. Considering the central role of Inflammation in general is a significant contributing and indispensable factor in wound healing and tissue repair. There might even be a possibility that advanced atherosclerotic lesions are beyond repair.

The patient centered studies in this thesis clearly show that we must reach beyond the available tools in the laboratory to probe the pathophysiology of atherosclerosis, and more urgently strive to bridge the gap to human disease²⁶. Special attention should go out to advanced atherosclerosis since this essential phase is missing in current models. Given the high prevalence of FCP in middle aged and elderly individuals, a relevant model would be one with neo-atherosclerotic plaque formation on an existing fibrotic calcified plaque. This type of lesion seems to develop without direct communication with the underlying adventitia and vaso vasorum and should therefore be considered a unique entity in the atherosclerotic process.

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