Identification of therapeutic targets in coronary artery disease: from patient to mice and back

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Summary, conclusions and future perspectives

Cardiovascular disease, together with cancer, remains the most important cause of death in Western societies. Although extensive research efforts have been made, many pathophysiological mechanisms remain incompletely understood, which hampers the development of new therapeutic drug targets. This thesis addresses two promising developments in the research on cardiovascular disease. The first part describes studies on the design of new biomarker signatures for the identification of high risk CVD patients, focussing on chemokine patterns in patients with manifest cardiovascular disease. Furthermore, experimental studies were performed to establish a causal involvement of identified chemokine markers in atherosclerosis related cardiovascular disease. In addition, leukocyte CETP expression was studied in patients with an acute coronary syndrome. The second part focuses on the effects of established risk factors such as aging and recently identified genetic mutations that correlate with CVD using mouse models and patient cohorts.

PART I: IDENTIFICATION AND ANALYSIS OF CHEMOKINES INVOLVED IN CARDIAC ISCHEMIA AND ATHEROSCLEROSIS

Chapter 2 provides an overview of the current status on chemokines and plaque stability to serve as starting point of the studies described in this thesis. Particular emphasis was put on available clinical data on chemokine expression in patient cohorts with cardiovascular disease. Various chemokines were shown to be associated with cardiovascular disease, ranging from stable angina pectoris to myocardial infarction and restenosis. The relevance of chemokines for CVD is supported by numerous experimental studies showing that intervention in chemokine or chemokine receptor expression in mouse models, as obtained by genetic or pharmacological strategies, can dramatically influence plaque size. However, only few studies show a direct effect on markers of atherosclerotic plaque stability. Thus, there is a clear need for additional studies which demonstrate the direct link of chemokine modulation and plaque stability parameters to augment development of future therapeutic chemokine-based therapies.

To evaluate specific chemokine signatures in acute coronary syndromes, we evaluated the expression of a panel of chemokines with a multiplex strategy in the APRAIS cohort (**chapter 3**). Not only did we detect increased circulating levels of CCL5 (RANTES) and CCL18 (PARC) during episodes of cardiac ischemia, the levels of these two chemokines were also higher in patients with refractory ischemic symptoms during hospitalization. The differences of chemokine release in refractory patients might be caused by enhanced and sustained platelet release of CCL5 and CCL18 upon renewed platelet activation in patients with refractory UAP or by enhanced release of these chemokines following myocardial ischemia. Furthermore, we studied chemokine receptor expression in circulating peripheral blood mononuclear cells (PBMCs) and specifically focussed on the receptors for CCL5 (CCR1, CCR3, CCR5) and CCL18 (CCR3) as well as CCR4. During ischemia, we detected increased levels of all 4 chemokine receptors during ischemia, pointing to enhanced signalling possibly by mobilisation of receptors. However, mRNA expression

levels were down-regulated, suggestive of a negative transcriptional feedback loop following chemokine receptor activation or mobilization of intracellular receptors in response to elevated serum ligand levels. The latter was substantiated in vitro, as PBMC incubation with synthetic CCL18 led to decreased CCR3 expression. Therefore, these studies led us to conclude that 1) CCL5 and CCL18 are transiently raised during ischemic symptoms, 2) CCL5 and especially CCL18 show potential of predicting future outcome in patient with an acute coronary syndrome and 3) leukocyte chemokine receptor expression is dramatically altered during periods of cardiac ischemia. It indicates that chemokines likely play a substantial role in the inflammatory post-ischemic response.

CCL18 might be a relevant chemokine in human cardiovascular disease, as a possible risk marker and/or as a chemokine directly regulating atherosclerosis. It is expressed at high levels in serum and is implicated in T-cell responses in other (inflammatory) diseases [1]. Data on CCL18 in atherosclerosis are however scarce, which is partly attributed to the fact that human CCL18 lacks a murine counterpart. In **chapter 4**, we have investigated its impact on lesion progression. First we analysed the expression of CCL18 in three stages of human atherosclerotic plaques by microarray and immunohistochemical staining. We observed a marked increase in CCL18 mRNA expression upon plaque progression. Furthermore, CCL18 protein expression was spread among the entire plaque. Next, we have investigated focal and targeted overexposure of CCL18 on plaque progression. Two strategies were applied to introduce human CCL18. First, we injected mice with synthetic CCL18 every other day intra-peritoneally for two weeks. After two weeks, CCL18 treated mice displayed increased atherosclerotic plaque size, without a clear effect on plaque morphology. This effect may be caused by CCL18 induced immune cell activation. Indeed, we observed aberrant splenic architecture in CCL18 treated animals, which might indicate release of inflammatory cells. Also, sCCL18 was seen to promote T-cell migration *in vitro*, although we did not observe any differences in plaque T-cell content. The most striking feature of chronic CCL18 treatment was the 40-fold increased serum IL-6 levels. Local overexpression of carotid artery plaques by luminal transduction with Ad-CCL18, creating a CCL18 gradient, yielded an increased plaque T-cell presence compared to Ad-empty controls. However, this strategy did not affect plaque size or other plaque characteristics. These data suggest that circulating CCL18 might act pro-atherogenic and/or attract T-cells to the atherosclerotic plaque in response to a local CCL18 gradient. The latter may lead to a T-cell mediated inflammatory cascade, aggravating plaque size and initiating plaque progression, potentially leading to plaque rupture.

In **chapter 5**, we sought to study the release of chemokines in patients with an acute myocardial infarction versus controls by a multiplex approach. This study was performed in a sub-cohort of the MISSION! intervention study. Levels of CCL3 (MIP-1α), CXCL8 and, again, CCL5 were significantly up-regulated upon myocardial infarction, even after adjustment for confounding factors. In contrast, levels of CXCL10 were reduced in AMI, illustrating the notion that chemokines may have divergent and even opposite functions during cardiac ischemia. As CCL5 and CXCL8 have already been linked to CVD and while data on CCL3 in ACS are scarce, we

focused in follow-up studies on the latter chemokine. Prospective analysis of CCL3 in the APRAIS cohort also showed elevated levels of CCL3 during cardiac ischemia compared with 6 months follow-up. Furthermore, elevated levels of CCL3 were associated with an adverse cardiovascular outcome as well. To assess if these differences were directly caused by the inflammatory ischemic response to cardiac ischemia, additional murine coronary ligation experiments were performed. These experiments confirmed our human observations, as elevated CCL3 levels were measured in the coronary ligation group compared to sham treated animals. Elevated levels of CCL3 coincided with an increase in circulating CCL3 responsive $CCR5^+$ T-cells, which suggest that the CCL3 burst might drive T-cell release and fluxes upon myocardial infarction. As these studies were performed in non-atherosclerotic mice, the data point to ischemia induced CCL3 release in patients with an acute coronary syndrome.

Next, **Chapter 6** describes our efforts in which we set out to elucidate the role of CCL3 in the onset and progression of atherosclerosis. Using a bone marrow transplantation model, we introduced CCL3 deficient leukocytes in LDLr deficient hyperlipidemic mice. CCL3 deficiency resulted in significantly lowered CCL3 levels upon injection with lipopolysaccharide as well as a decreased atherosclerotic plaque formation compared to controls. Furthermore, neutrophil adherence and content in CCL3 deficient mice was markedly reduced, and LPS administration to CCL3-/- mice resulted in significantly decreased levels of circulating neutrophils. These data indicate that under conditions of acute inflammation leukocyte derived CCL3 can induce neutrophil chemotaxis towards the atherosclerotic plaque, thereby accelerating lesion formation.

Recently, the class of CETP-inhibitors has gained a lot of negative attention. The ILLUMINATE trial was stopped because of an increased mortality rate in the Atorvastatin + Torcetrapib treated group, leading to the withdrawal of Torcetrapib from the market and hampering further developments of other CETP inhibitors. Reasons for this increased mortality were off-target effects of Torcetrapib, concerning increased sodium and aldosterone levels, with a concomitant increase in blood pressure. Furthermore, more patients in the Torcetrapib group died of infections. In **chapter 7**, we therefore set out to study the relationship between leukocyte CETP expression and acute coronary syndromes in the APRAIS cohort. We detected a 3-6 fold down-regulation of leukocyte CETP mRNA and protein expression during an acute coronary syndrome compared to 180 days of follow-up. This was corroborated in a coronary ligation experiment in CETP transgenic mice. Leukocyte CETP expression was down-regulated in mice with a myocardial infarction compared to sham treated mice, which correlated strongly with increased SAA inflammatory activity. These latter findings indicate that reduced CETP activity is associated with an enhanced inflammatory status. This increased inflammatory state may be beneficial in an acute coronary syndromes and post-ischemic responses, but may become a problem when CETP activity is chronically suppressed with CETP inhibitors. Therefore, future CETP inhibitors should be meticulously tested for their effects on the immune system before they are studied in large-scale clinical trials.

CONCLUSIONS AND FUTURE PERSPECTIVES, PART I.

The studies described in the first part of this thesis expand our knowledge on the influence of chemokines in ACS as well as atherosclerosis next to the relationship of leukocyte CETP expression and ACS. These studies fuel the notion that chemokines could potentially be used as risk markers and/or as therapeutic targets in cardiovascular disease. We provide further data that chemokines are essential actors in the development and progression of atherosclerosis. Our studies add CCL3 and CCL18 to the expanding number of chemokines shown to be critically involved in this process, possibly by promoting the recruitment of neutrophils and T-cells to the plaque. Although additional studies are needed to pinpoint the exact mechanism of their pro-atherogenic activity, our studies indicate that targeting these ligands might be an avenue worth exploring in the development of suitable drug targets. As chemokine or chemokine receptor inhibition may come with potentially adverse side effects due to derangement of the immune system, local application seems to be the most elegant way to target the atherosclerotic plaque. Nevertheless, systemic chemokine inhibition strategies have already been tested and proved effective in for instance HIV patients while specific anti-atherosclerotic CCR2 antagonists are currently under development.

Two important additional questions are (1) whether elevated chemokine levels are cause or consequence in the pathogenesis of an acute coronary syndrome (ACS) and (2) whether they can serve as biomarkers for prognosis in patients with an ACS. As described in this thesis and in keeping with other studies, levels of circulating chemokines are elevated during cardiac ischemia, in response to tissue injury and/or thrombosis, leading to the recruitment of dedicated leukocyte subsets to the infarcted area. Our multiplex analyses show that not all chemokines are differentially expressed upon ischemia however, suggesting that some chemokines do not take part in this process at all. Furthermore, we cannot exclude that baseline levels of CCL3, CCL5 or CCL18 were already elevated in individuals with atherosclerosis and unstable plaques at high risk of plaque rupture and ischemia.

Regarding the second question our studies clearly indicate that elevated levels of CCL3, CCL5 and CCL18 in ACS patients predispose to recurrent ischemic symptoms or revascularization in the future. Therefore, these chemokines could serve as biomarkers or risk predictors, although our studies were performed in relatively small cohorts. Obviously larger sized studies with more statistical power are needed to validate these preliminary, promising results and to firmly establish the observed associations with hard cardiovascular end-points. Also, we have to keep the caveats of chemokine detection in serum or plasma in mind. For instance, repeated freeze-thaw cycles may negatively affect chemokine stability leading to diminished chemokine detection, plasma levels of some exotic chemokines may be close to or even below detection limits of our multiplex immuno-assays and chemokines could be released ex-vivo from primed platelets thereby generating artefactual elevation of in-vivo levels [2]. Furthermore, a general inflammatory response might also give rise to elevated chemokine levels, which makes chemokine assessment in patient with cardiac ischemia and concomitant inflammatory disease

or infection difficult and less specific. Finally, reliable standardized tests need to be developed in order to acquire reproducible measurements in various patient groups. Clearly, a lot of work needs to be done in order to take chemokine measurements from bench to bedside.

Nevertheless we believe that our multiplex strategy for detecting signature rather than individual chemokine and cytokine markers may be an interesting option for additive future risk assessment. However, a multiplex test to identify individuals with increased risk or poor prognosis has to be superior regarding for instance sensitivity, specificity, cost and speed to current markers such as troponin-T or NT-proBNP. At present the high costs as well as limited current validation in CVD disqualifies chemokine detection currently in the direct routine clinical setting. Further pre-clinical research and advanced technical developments may translate into clinical application.

PART II: ATHEROSCLEROSIS ASSOCIATED GENES: FUNCTION IN ATHEROGENESIS AND PLAQUE STABILITY

In **chapter 8**, we studied the effect on atherogenesis of a high fat diet in young and aged LDLr-/- mice. Both mouse groups were put on a high fat diet for an identical period of time, after previously being fed with a chow diet. Hereby, we tried to circumvent the problem of a life-time exposure to pro-atherogenic stressors normally experienced in human life. To our surprise, aged mice displayed a decreased plaque size, both at the carotid artery as well as the aortic root level. In contrast, their ability to show an outward arterial remodelling response was hampered, resulting in an increased luminal stenosis. To elucidate some of the genetic pathways which regulate vascular remodelling, we performed a micro-array experiment in carotid arteries of these mice. We detected approximately 40 genes which were significantly up- or down-regulated upon aging. We went on to study one of these genes, Quaking, as this gene was shown to be involved in embryonic vascular development. Not only did we detect Quaking protein expression in murine and human atherosclerotic tissues, temporal expression analysis of carotid arteries revealed a striking up-regulation of Quaking with vascular aging, suggesting an involvement of Quaking in adult vascular homeostasis.

Therefore, we went on to study the role of Quaking in vascular smooth muscle cell (VSMC) function in **chapter 9**. First, the influence of 12 Quaking single nucleotide polymorphisms (SNPs) was determined in patients with clinical restenosis following a percutaneous coronary intervention in the GENetic DEterminants of Restenosis (GENDER) study. Out of these 12 SNPs, 4 were significantly associated with an increased rate for the need of target vessel revascularisation (TVR), whereas 3 SNPs were seen to have a protective effect. Furthermore, the combination of the protective 57896AA and the 65752 AG and GG risk-alleles, present in 10% of the population, resulted in a hazard ratio of 1.77 for TVR. Next, the effects of Quaking abrogation in VSMCs were studied with the use of Quaking viable mutant mice. These studies demonstrated

a remarkable VSMC dysfunction upon Quaking down-regulation. Also, a femoral artery neo-intimal hyperplasia mouse model in these mice demonstrated a significant decreased neo-intimal proliferative response in conjunction with a decreased amount of VSMCs present within these lesions. These data indicate that Quaking might be used as 1) a genetic marker to identify patients at high risk for restenosis and 2) as a therapeutic anti-restenotic target.

Over the past years, the influence of the Myocyte Enhancer Factor 2A (MEF2A) gene as a risk factor for acute myocardial infarction has been heavily debated. The initial publication on a genetic linkage analysis, demonstrating an increased risk for myocardial infarction within one family caused by a seven amino acid deletion in the MEF2A gene, was followed by various population studies revealing inconsistent results on the influence of MEF2A in myocardial infarction. In **chapter 10**, we set out to study the role of the entire MEF2 gene in atherosclerosis and endothelial cell function. Using a local adenoviral gene transfer strategy in murine carotid artery plaques, adenoviral directed dominant negative MEF2 expression induced plaque erosion compared to control vectors, as demonstrated by decreased endothelial cell presence, resulting in an enhanced intra-plaque bleeding. Furthermore, endothelial cell function was reduced in vitro as evidenced by decreased proliferation and matrigel spouting upon infection with dominant negative MEF2. Finally, micro-array analysis in endothelial cells revealed overlaps of MEF2 with VEGF signalling pathways.

Although the latter observation is explorative and indicative for future research, MEF2 down-regulation induces endothelial cell dysfunction, thereby augmenting plaque erosion and/or intra-plaque haemorrhage. This points to a causal role of MEF2(A) dysfunction and the risk for myocardial infarction, thereby showing that further research is needed to elucidate its definitive role in the pathogenesis of myocardial infarction.

CONCLUSIONS AND FUTURE PERSPECTIVES, PART II.

The studies described in the second part of this thesis discuss the effects of aging and genetic mutations on various aspects of cardiovascular disease, with emphasis on functional testing of gene associations with myocardial infarction and restenosis. The Quaking gene which emerged from our aging experiments was further characterized regarding VSMC function. Quaking transcriptional activity seems to be vital for proper VSMC plasticity, *in vitro* as well as *in vivo*, which makes it an interesting genetic target for future drug therapies. A strategy to modify Quaking transcriptional activity would be via local controlled delivery of a Quaking antagonist or decoy via coronary stenting. This might slow uncontrolled neo-intimal proliferation which leads to restenosis. However, regulators of Quaking activity are currently still unclear. Furthermore, as Quaking has an influence on a wide variety of cells and cellular functions [3], Quaking down-regulation should be handled with care and we recommend at this stage local rather than systemic application.

The association of MEF2 with atherosclerosis and myocardial infarction remains a heavily debated issue. The initial study by Wang et al. on an association of MEF2A with myocardial infarction has never been confirmed in other larger scaled genetic population analysis [4-6] . The question is therefore legitimate if Quaking, MEF2A or any other genetic association with cardiovascular disease are useful for individual cardiovascular risk prediction. In this regard, the studies described in part II are important as they give further insights into the exact mechanism by which Quaking and MEF2 exert their function in cardiovascular disease. Our results plead for a causal role of MEF2 family members in the maintenance of endothelial patency, and support the notion that MEF2 dysfunction could promote plaque erosion and ensuing thrombosis. However, many other cardiovascular genetic associations still lack a functional understanding. Genome-wide association studies are yielding a rapidly increasing number of genes associated with CVD but actual improvement in cardiovascular risk prediction is still limited [7]. This poor utility is a very relevant feature, as commercial arrays are currently available to determine an individual genotype. These diagnostic measures will hypothetically allow the physician to not only identify high risk patients that require extra care, but also will bring within reach the option of tailor-made therapy as a particular genetic signature will likely give information on the most effective and well tolerated therapy for that patient. It is expected that these initiatives will only increase in the near future. Therapies based on gene profiling are however not yet available for cardiovascular disease, but patients receiving breast cancer treatment are currently stratified to various categories of drug therapies based on the molecular classification of malignant cells. Additional specific gene expression signatures are already used to guide clinicians in clinical decision making [8]. This knowledge is primarily gathered via massive genetic analyses of genome-wide expression patterns in breast cancer cells [9]. As previously discussed, various studies have been performed which use genomic strategies to assess gene expression in atherosclerotic plaques [10, 11]. It seems only a matter of time until the first treatment strategies based on these results will enter the stage of clinical testing. The bulk of data which are rendered through genome-wide association studies are likely to pay off in the future, as many new therapeutic targets are being identified, targets which can prevent or regress atherosclerosis and its clinical consequences. These targets could be specific genes, genetic patterns or proteins. In the long run, the former two could be the most appropriate targets like in breast cancer patients, as genetic profiling is being applied on an expanding scale with advanced techniques and even reduced costs. One has to keep in mind that although we have these powerful analytic tools in hand, genetic profiling would only be feasible with a concomitant broad range of therapeutic options, ranging from specific drugs to vascular devices or diets. The future seems to look bright from a cardiovascular point of view. However, both protein and mRNA expression are deeply influenced by environmental factors and a detrimental lifestyle, which both have an independent association with the occurrence of cardiovascular disease. Furthermore, we have to realize that the expression of genes and proteins is not organized in a 1:1 relationship. Therefore, preventive strategies to reduce cardiovascular disease remain the cornerstone of current and future cardiovascular care.

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