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Chapter 8

General Discussion and Perspectives
8.1 Introduction
Cardiovascular disease (CVD), which encompasses a group of disorders of the heart and vasculature, remains a leading cause of death world-wide. Currently, about 17.3 million people die annually as the consequence of CVD and with the growing population and increase in life expectancy this number has been estimated to grow to more than 23.2 million a year by 2030. In the majority of these cases death is caused by a myocardial infarction or a stroke due to thrombotic blood vessel occlusion as a consequence of atherosclerotic plaque rupture or erosion. Atherosclerosis is the underlying lipid-induced chronic inflammatory disease, which already starts early in life and progresses until clinical symptoms may become apparent, often from the age of 60 and onwards. In addition to the individual grief, the economic burden on society is enormous, totaling 320 billion a year (in the US) due to health expenditures and lost productivity. The main therapeutic strategy is based on lipid lowering by means of statin treatment, which are HMG-CoA reductase inhibitors, a rate limiting enzyme in cholesterol synthesis. Under current guidelines, patient’s cholesterol levels are aimed to be lowered to a considered healthy <70 mg/dL (<1.8 mmol/L), however recent trials seem to indicate that even further lowering (for example by combination therapy with Ezetimibe) results in additional risk reduction. In addition to its lipid lowering potential, statins have been shown to exhibit anti-inflammatory properties, which combined have reduced the relative risk of CVD-related deaths in patient by 25-30%. Nonetheless, there remains a considerable residual risk and the absolute risk reduction, especially in patients with less extreme cholesterol levels, is limited. This clearly emphasizes the need for new therapeutic modalities based on a more thorough understanding of the disease and all its risk factors.

Besides dyslipidemia and traditional risk factors for cardiovascular disease such as smoking, untreated hypertension, physical inactivity and diabetes mellitus, a contributing role for psychosocial risk factors, including psychological stress, has become evident and inspired research into the biological pathways involved.

8.2 Psychological stress: an underappreciated risk factor for cardiovascular disease
Early work by Rosenman et al. indicated a clear correlation between certain behavior and personality types and the risk of developing coronary heart disease. These studies provided the first link adverse between emotional states, which can be considered in general as stressful, and disease. Further evidence for a direct role for psychological stress in the development of atherosclerosis was provided by landmark studies in cynomolgus monkeys by Kaplan et al. demonstrating extensive coronary atherosclerosis in stressed animals compared to their unstressed controls, even under normocholesterolemic conditions. More recently,
the association between multiple psychosocial risk factors and the incidence of acute myocardial infarction (AMI) was assessed in the large case-control study INTERHEART. Especially chronic exposure to for example work-related stress correlated with a more than 2-fold greater risk of AMI.

Chapter 2 of this thesis provides an overview of the major biological systems known to be involved in stress-induced exacerbation of cardiovascular disease, including the inflammatory response, the autonomic nervous system, neuroendocrine and oxidative systems. Both human epidemiological and clinical data as well as mechanistic insights obtained from various animal models are discussed. A clear distinction is made between chronic and acute stress exposure. While chronic stress results in the wear-down and maladaptation of the various systems, including the immune system, acute stress boosts various responses via the central and local release of stress-related hormones and neuropeptides. Additional attention is given to the role of early life stress as atherosclerosis development initiates already in childhood and adolescence, necessitating earlier intervention strategies.

8.3 Stress-induced plaque vulnerability and atherothrombosis: a key role for mast cells?

In Chapter 3 and 4, the immunomodulatory potential of the acute stress response in relation to atherosclerotic lesion progression, vulnerability and atherothrombotic complications were investigated. Previous research had uncovered a pro-atherogenic effect of the sympathetic nerve-derived factor substance P, acting in part via mast cell activation, and a strong colocalization between nerve fibers and mast cells in the perivascular tissue. This inspired us to investigate the more general acute stress response in modulating mast cell activity with regard to atherosclerotic plaque vulnerability and atherothrombotic complications in Chapter 3. To establish whether acute stress, in this case a 30-120 minute period of restraint stress, was capable of activating perivascular mast cells, apoE−/− mice were subjected to the stress protocol and their stress response monitored. As expected, acute restraint caused a strong rise in plasma corticosteroid levels, but also resulted in a significant increase in circulating amount of the pro-inflammatory cytokine IL-6. Subsequent morphological characterization of various (vascular) tissues, demonstrated especially a significant increase in cardiac mast cell activation after 120’ of restraint stress exposure. In addition to increased atherosclerosis development upon chronic mast cell activation, pro-inflammatory cytokine and protease release from mast cells at later stages of the disease was shown to decrease atherosclerotic lesion stability. In humans, acute exposure to severe stressors, such as the terror of a natural disaster or terrorist attack and the intense grief after losing a loved one, is highly linked to increased risk of myocardial infarction or stroke. To test the hypothesis, that acute stress-induced mast cell activation contributes to this we evaluated the effect of acute stress-induced mast
cell activation on atherosclerotic lesion stability. ApoE⁻/⁻ mice were put on a high fat diet for 6 weeks to induce the development of advanced atherosclerotic lesions in the aortic root. Next, the mice were subjected to 120’ restraint stress with or without prior administration of the mast cell stabilizer cromolyn. Subsequent analysis of the collagen content and the incidence of intraplaque hemorrhages (IPH) in the atherosclerotic lesions, both hallmarks of a vulnerable plaque in mice, demonstrated a significant reduction in collagen in the stressed mice compared with non-stressed controls. Furthermore, the incidence and size of the IPH were higher in the stressed mice, possibly due to increased leakiness of intraplaque neovessels as previously shown for other routes of mast cell activation. The contribution of the mast cell herein was confirmed by the inhibition the stress-induced effects on systemic inflammation and locally on plaque vulnerability by pretreatment with cromolyn. However, the inhibition was only partial, which might be explained by the relative poor effectiveness of cromolyn in mice. To further confirm the mast cell-dependency of the observed pro-atherogenic effects of acute stress, a similar experiment was performed in mast cell depleted apoE⁻/⁻ mice. In line with the results obtained by cromolyn pretreatment, mast cell deficiency significantly reduced the stress induced increase in circulating levels of IL-6 and completely abolished the effects of stress on plaque vulnerability parameters.

The majority of cardiovascular disease-related deaths are caused by the thrombotic occlusion of coronary or cerebral arteries, resulting in a myocardial infarction or stroke. Rupture or erosion of the fibrous cap, of an advanced and vulnerable atherosclerotic lesion exposes the thrombogenic content of the plaque and initiates platelet activation and the blood coagulation system. In Chapter 4 the direct contribution of the acute stress response on platelet activation, clot formation and thrombus formation was assessed. Although 120’ restraint stress in apoE⁻/⁻ mice resulted in a robust decrease in circulating white blood cells and a redistribution of the different leukocyte subpopulations, red blood cell and platelet numbers were not changed. Furthermore, platelet activation status, assessed by agonist induced expression of the glycoprotein αIIbβ3, was not affected. However, acute stress did increase tail bleeding time and somewhat reduced clot retraction capacity, both indicative of impaired coagulation. Next, we investigated the direct effect on thrombus formation, by means of a FeCl₃-induced carotid artery thrombosis model. Topical application of FeCl₃ in this model results in rapid endothelial damage, platelet activation and adherence to the vessel wall and subsequent formation of a vessel occluding clot. Interestingly, exposure to acute stress, either directly or 24 hours upfront did not affect the time to occlusion of results in any obvious differences in the composition of the thrombi. Similar experiments in the mast cell deficient apoE⁻/⁻ mice did not reveal mast cell-dependent effects, except an inhibition of the acute-stress induced increase in tail bleeding time. Further research is necessary to dissect out the potential mast cell-derived mediators.
implicated in this effect. Combined, the results described in chapter 3 and 4, indicate the acute stress response as a potent mast cell activator, resulting in increased inflammation plaque destabilization. With regard to atherothrombotic complications and the direct effect of acute stress on thrombus formation, no clear results were obtained necessitating additional research. Possibly targeting specific mast cell-derived mediators, such as heparin could shed light on the observed difference in tail bleeding time.

8.4 Neuropeptide Y signaling in atherosclerosis
One of the most abundantly expressed stress-related neuropeptides is neuropeptide Y (NPY). After its identification in porcine brain extracts in 1982\textsuperscript{11}, further research determined the widespread distribution of this 36-amino acid peptide both in the central and peripheral nervous system. Peripherally NPY is co-stored and co-release with norepinephrine and ATP and potentiates the effects of these neurotransmitters. The first biological function identified was its potent vasoconstrictive property on cerebral and renal arteries, making NPY a possible drug target for the prevention of hypertension. However, additional research elucidated strong mitogenic effects of NPY on vascular smooth muscle cells, endothelial cells and adipocytes\textsuperscript{12}, and provided associations between chronic stress-induced increases in NPY levels and obesity and metabolic syndrome.\textsuperscript{13} The direct involvement of NPY in atherosclerosis was inferred from increased disease progression in patients with a gain-of-function mutation in the preproNPY gene.\textsuperscript{14} In Chapter 5 the expression of NPY in stable versus unstable human and murine atherosclerotic lesions was investigated. NPY expression in carotid endarterectomy specimens obtained from the AtheroExpress biobank\textsuperscript{15} was significantly higher in unstable compared with stable lesions. In line with these results, increased expression was also observed during atherosclerosis progression in apoE\textsuperscript{-/-} mice. To establish a direct pro-atherogenic effect of NPY and investigate the local effect increased perivascular concentrations of NPY, for instance after local release from adventitial nerve fibers, we constructed a NPY-expressing lentivirus for perivascular application at the site of atherosclerotic lesion formation. Rapid atherosclerosis development at an accessible site was induced by collar-placement around the carotid arteries as described by von der Thusen et al.\textsuperscript{16} Next the NPY-lentivirus was applied in pluronic gel to ensure the local overexpression. In line with previous results, overexpression of NPY resulted in a significant increase in lesion size. Interestingly, perivascular mast cell activation was also significantly increased, leading us to evaluate NPY-induced mast cell activation. In vitro incubation of bone marrow-derived mast cells with recombinant NPY indicated a bimodal response, resulting in the release of the pro-inflammatory cytokine IL-6 and mast cell-specific protease tryptase by NPY.
concentrations in the high μM and low nM range. NPY acts both centrally and peripherally through its G-protein couple receptors (Y1-Y6). Of these receptors, Y1, Y2 and Y5 are most ubiquitously expressed and best characterized. In Chapter 6 we obtained the expression profiles of these different receptors during atherosclerosis and restenosis in apoE−/− mice. In contrast to NPY itself, which was increasingly expressed in both disease models, expression of the Y1 receptor was almost completely abolished upon disease initiation. In contrast expression of the Y2 and Y5 receptors as well as the peptidase DPPIV, which cleaves NPY in NPY3-36 lacking Y1 receptor affinity, was generally higher during atherosclerosis progression. Next we assessed the therapeutic potential of systemic Y1, Y2 or Y5 antagonist treatment in atherosclerosis. LDLr−/− mice put on WTD were injected 3 times a week with a specific Y1 (BIBO-3304), Y2 (BIIE-0246) or Y5 (CGP-71683) receptor antagonist for a 6 week period. In contrast to previous results obtained in restenosis models in rats and mice in which NPY- or stress-induced neointima formation could be inhibited by both local and systemic Y1 receptor antagonism, atherosclerotic lesion formation was increased. Especially, Y2 receptor antagonism led to a faster disease progression resulting in significantly bigger and more advanced lesions, with lower macrophage and vascular smooth muscle cell content and increased perivascular mast cell accumulation. Interestingly, pro-atherogenic changes, upon altered Y1 signaling, including changes in food intake and triglyceride metabolism17,18 and increased levels of the pro-inflammatory cytokine IL-1219 could be observed in the Y1 and Y5, but not the Y2 receptor antagonist treated mice.

The results from Chapter 3 and 5 more firmly established the important contribution of mast cells in atherosclerosis development and progression of plaques towards a vulnerable state. Packed with pro-inflammatory mediators and uniquely localized to respond quickly to pathogens, but also endogenous danger-associated signals and neurogenic stimuli, these innate immune cells contribute to the ongoing vascular inflammation and can reduce the integrity of the plaque.20 Besides these pro-inflammatory cytokines and proteases, mast cells secrete chemokines such as MCP-1 and IL-8, which can modulate the influx of other immune cells. In Chapter 7, we investigated the recruitment of leukocytes towards the atherosclerotic lesion upon systemic mast cell activation. To control for a previously observed increase in circulating levels of IgE in Western type diet (WTD) fed apoE−/− mice, we performed the mast cell activation in apoE−/− μMT mice, which lack endogenous IgE. Interestingly, besides a faster lesion development, systemic mast cell activation, by means of antigen-induced FcεR crosslinking, during the 8 weeks on WTD resulted in a striking increase in intimal perivascular neutrophil recruitment. To dissect out the mast cell-specificity of this effect, peritoneal influx studies in C57Bl/6 and mast cell deficient KitW−/−/W−/− were performed. Peritoneal injection of the mast cell activator compound 48/80...
resulted in a significant increase in, especially the chemokine receptor CXCR2 and CXCR4 positive, neutrophils in the control apoE<sup>−/−</sup> mice but not in the mast cell deficient mice. In vitro migration assays, using a transwell system in which isolated neutrophils were shown to migrate towards activated mast cell supernatant, demonstrated the primary involvement of the CXCR2 receptor on the neutrophil, as CXCR2 blockade, but not CXCR4 receptor antagonism could inhibit the migration. This was further confirmed in vivo were pretreatment with an anti-CXCR2 receptor antibody similarly blocked compound 48/80 induced peritoneal recruitment of neutrophils. These data provide an additional mechanism by which these two innate immune cells reinforce each other’s responses and thereby aggravate the ongoing vascular inflammation leading to accelerated atherosclerosis.

**Considerations and perspectives**

Cardiovascular diseases in humans develop over decades and clinical symptoms generally manifest from the age of 60 onwards. However, the vascular damage and inflammation associated with atherosclerosis initiation already occurs during early adolescence, resulting in a complex disease course modulated by many different risk factors throughout the lifetime. Psychosocial risk factors, including psychological stress, have gained well-deserved attention as important (modifiable) risk factors contributing to the metabolic, endocrine and inflammatory processes involved in atherosclerosis.

In this thesis, the immunomodulatory properties of the acute stress response and the neuronal hormone NPY in the context of atherosclerosis development and progression have been investigated. Special attention was given to the contribution of the mast cell herein, and modulation of its activity by the stress response. Our data provide evidence for a direct contribution of acute stress-induced mast cell activation in atherosclerotic plaque destabilization. Also, increased vascular expression of the stress-related hormone NPY was shown to correlate with disease severity and contribute to atherosclerosis development, at least partly, via the induction of perivascular mast cell activation. Combined with the novel insights in mast cell-mediated modulation of other immune responses involved in atherosclerosis, such as neutrophil recruitment to the plaque, therapeutic strategies aimed at mast cell inhibition or stabilization seems a valuable approach for the prevention of cardiovascular disease-related deaths. Up to date, few studies evaluating the cardiovascular benefits of mast cell stabilization, for example by means of anti-allergic drugs, have been performed. The Prevention of RESTenosis with Tranilast and its Outcomes (PRESTO) trail, assessed the anti-inflammatory and anti-proliferative properties of the anti-allergic drug tranilast for the prevention of restenosis after percutaneous coronary intervention. Unfortunately, in contrast to two smaller previous trials<sup>21</sup>, no improvement in angiographic or clinical restenosis was observed in this multicenter randomized clinical trial.<sup>22</sup>
While tranilast has been shown to inhibit pro-inflammatory mediator release from mast cells it also effects proliferation and migration of smooth muscle cells and fibroblasts complicating the evaluation of mast cell specific effects. More recently, several patents regarding the use of mast cell stabilizers for the prevention and treatment of cerebral ischemia\textsuperscript{23}, cardiovascular disease in general\textsuperscript{24}, obesity\textsuperscript{25} and in combination with statins for inflammatory disorders\textsuperscript{26} were filed. These patents highlight the potential of mast cell stabilization in a variety of disease. However, systemic mast cell inhibition might compromise the protective responses of the mast cell as part of the immune system, in for example the skin, lung and intestine, against bacterial and parasitic infections. Intervention strategies, aimed at modulating mast cell function and activation via disease-specific ligands might circumvent this while retaining the desired beneficial effects. One such example might be inhibition of mast cell activation by specific complement factors highly expressed in vein graft disease.\textsuperscript{27} With regard to mast cell triggers in the context of atherosclerosis development and progression, we identified increased vascular NPY expression as a potential mast cell activator, leading to increased disease progression. However, our results also indicate the difficulty of modulating NPY signaling, as its receptors are expressed both in the central nervous system and the periphery acting on various endocrine, metabolic and immunologic responses. Also systemic application of for example Y1 receptor antagonists did not influence exercise-induced ischemic parameters in patients with coronary artery disease\textsuperscript{28} and Y5 receptor antagonism did not augment the weight loss efficacy of two anorexiants, orlistat and sibutramine.\textsuperscript{29} Animal studies, especially with regard to restenosis, demonstrated clear beneficial effects of Y1 receptor antagonism and one might envision local application of such antagonists by means of drug eluting stents. Furthermore, the Y2 receptor was demonstrated to be indispensable in mediating stress-induced obesity and metabolic syndrome. Up to date no conclusive results demonstrating a beneficial effect of Y2 receptor antagonism in humans has been obtained and our data (in mice) indicate that systemic Y2 receptor antagonism, like previously shown for Y1 receptor antagonism\textsuperscript{19}, may actually promote atherosclerotic plaque development via a currently unresolved mechanism. Furthermore, improved glycemic control in type 2 diabetes mellitus patients treated with the DPPIV inhibitory sitagliptin, which besides the degradation of the gastrointestinal hormones GLP-1 and GIP, also modulates NPY signaling and has been associated with increased heart failure.\textsuperscript{30} However, a recent large, multi-center trial evaluating such detrimental side-effects of sitagliptin in addition to usual care, did not show significant differences in hospitalization rates for heart failure, acute pancreatitis or pancreatic cancer\textsuperscript{31}, warranting further mechanistic insight into NPY signaling and ways of harnessing the therapeutic potential of these specific NPY receptor antagonist for the prevention of cardiovascular disease. As mentioned before, the psychological stress response contributes to
cardiovascular and metabolic disease a.o. via the upregulation of neuropeptide Y. Furthermore, other major stress hormones, including glucocorticoids, adrenalin and noradrenaline have all been implicated in the correlation between risk of cardiovascular complications and chronic exposure to psychosocial risk factors, such as work-related stress and depression. Several trials have addressed the cardiovascular benefit of psychological treatment of depression, including the SADHART, ENRICHED and MIND-IT trials32, but unfortunately without clear effects on cardiovascular disease. In addition to atherosclerosis progression, plaque vulnerability remains a key determinant of atherothrombotic complications. Here, we identified a direct link between acute stress exposure and plaque destabilization through increased mast cell activation. Combined with acute stress induced changes in coagulation, these results warrant further investigation into the specific mediators involved and therapeutic potential of modulating both the stress response and immune cell activation. Such therapeutic interventions might best focus on the prevention of secondary events in patients with confirmed vulnerable lesions.

In conclusion, the stress response, a.o. acting via the synthesis and release of various neuropeptides, significantly contributes to atherosclerosis development and constitutes a relatively underappreciated risk factor for acute cardiovascular syndromes. The results obtained in this thesis provide further evidence for the direct influence of the stress response in modulating innate immune responses which contribute to atherosclerotic plaque development and destabilization. Future (clinical) investigations evaluating and treating mental health combined with lipid-lowering and anti-inflammatory strategies may be the necessary next step in the prevention of cardiovascular disease.

References

23. Use of a mast cell activation or degranulation blocking agent in the manufacture of a medicament for the treatment of cerebral ischemia. at <http://www.google.com/patents/WO2004071531A1>