Drainage versus defense

Valorization

Valorization

Social and economic value of the current thesis

Cardiovascular disease (CVD) is the most costly class of diseases, in comparison to other major diagnostic groups. Thus it accounts for an immense global health and economic burden [1]. A staggering 17 million people worlwide die annually from myocardial infarction and stroke (www.who.org). In the United States, CVD was responsible for an estimated health expenditure of around $316.1 billion in 2012 and 2013 [1], and projections are even more alarming, with total costs expected to increase by 117 % between 2012 and 2030 [1]. Atherosclerosis, specifically the rupture of an atherosclerotic plaque, is the main underlying cause of this tremendous cardiovascular mortality and morbidity [2]. Despite pharmacological advancements, no therapy is presently able to fully eradicate atherosclerosis or prevent plaque rupture [3].

The studies contained within this thesis investigated novel therapeutic options in CVD, focusing on (I) heart failure as a consequence of pressure overload, and (II) atherosclerosis. Subsequently, we have demonstrated that (I) the small molecule AVE3085 helps to maintain eNOS function, and thus may improve eNOS/NO signaling-dependent cardiovascular function, and (II) TNIP2 induction is associated with lymphangiogenic responses in the atherosclerotic plaque, while TNIP2 knockdown prevented normal lymphangiogenic development *in vitro* and in zebrafish *in vivo*. This secondary finding implies that TNIP2 induction could improve lymphatic drainage of constituents, cytokines and inflammatory cells from human plaques, thus potentially dampening atherosclerosis progression. These findings are relevant both to healthcare providers and to individuals at risk for CVD. Furthermore, the knowledge acquired here will directly contribute to the expertise of the cardiovascular researchers involved, allowing them to continue to advance our understanding of the molecular mechanisms of CVD. While this thesis presents data from basic research, and is not applicable for direct translation into clinical practice, it nevertheless provides a novel starting point for future developments in cardiovascular research, and potential for the development of new therapies.

Current and future treatment of heart failure

Classification of heart failure (HF) is based on left ventricular ejection fraction (EF). A reduced EF in patients with clinical signs and symptoms of HF is referred to as, HF with reduced ejection fraction (HFrEF). However, more than 50 % of all HF patients exhibit HF with preserved ejection fraction (HFpEF). These patients are predominantly elderly women, and have high rates of associated comorbidities, such as obesity and hypertension [4, 5]. Unlike HFrEF, the diagnosis of HFpEF is difficult; being easily missed by echocardiography - especially in patients who often show

normal EF, have multiple comorbidities and have no obvious physical signs of fluid overload [6, 7] .

HF cannot be cured, but it can be treated. The goal of treatment in patients with HF is to improve their clinical status, functional capacity and quality of life, preventing hospital admission and reducing mortality [8, 9]. However, life prolongation dependents upon the inhibition of cardiac remodeling [10]. Anti-neuroendocrine treatment (i.e. angiotensin converting enzyme inhibitors, mineralocorticoid receptor antagonists, and beta-blockers) has been shown to reverse modeling, and is proven to be effective in treating patients with HFrEF. In contrast, modern heart failure pharmacotherapy has not been shown to improve outcome in HFpEF [4, 9, 11]. As such, there is a great unmet need for new therapeutic approaches for HFpEF. It has been well documented that oxidative stress, decreased NO bioavailability and dysfunctional eNOS (uncoupled eNOS) contribute to myocardial remodeling and dysfunction in HFrEF and HFpEF [12, 13]. This indicates that maintaining or restoring eNOS functionality, subsequently increasing NO bioavailability, is a promising therapeutic intervention in HF.

Perspectives on AVE3085

In chapter 5, we have demonstrated that AVE 3085 attenuates the pathological left ventricular changes caused by pressure overload. AVE 3085 enhanced eNOS function by restoring its coupling ability, and subsequently reducing myocardial reactive oxygen species (ROS) generation. This effect most likely results from increased protection of BH4 from ROS-mediated oxidation. In addition, AVE3085 has been shown to ameliorate diastolic dysfunction and reverse cardiac remodeling in DAHL; a salt sensitive rat model of HFpEF [14]. These findings create new opportunities to use the eNOS/ NO pathway as a therapeutic target in the treatment of HF.

AVE3085 may have advantages over several other eNOS modulators. For instance, administration of BH4 has been shown to improve systolic and diastolic function in experimental HF [15, 16] . However, BH4 has a relatively narrow dose–response window, which remains a point of concern for the translatability of BH4 supplementation for clinical disease [17, 18]. Upon oral administration, BH4 is largely oxidized to BH2, which can then be re-reduced once inside the cell. This conversion may be limited by diseases with oxidative stress [19]. Moreover, higher doses of BH4 might tip the balance toward more BH2, which can then compete with BH4 to impair eNOS coupling, leading to a paradoxical reversal of benefit [17, 18]. This suggests that alternative pharmacological approaches to prevent BH4 oxidation or increase BH4 biosynthesis may be a more rational therapeutic strategy to improve eNOS functionality [20]. This can potentially be accomplished by AVE3085 alone, or in combination with other eNOS modulators; restoring BH4 bioavailability to

therapeutically perturb nitroso-redox balance in cardiovascular disease. For instance, folic acid and its metabolically active form 5-methyltetrahydrofolate (5-MTHF), increased binding affinity of BH4 to eNOS, and enhanced the regeneration of BH4 from its inactive form dihydrobiopterin (BH2) [21, 22]. Moreover, Vitamin C or L-ascorbic acid have been shown to stabilize BH4 [23]. Clearly, additional studies are needed to further clarify the potential role of AVE3085, with or without co-supplementation, for the treatment and/or prevention of cardiovascular disease. In future studies, I would determine the effect of AVE3085 in eNOS-deficient mice to confirm that AVE3085 is indeed eNOS-dependent. Furthermore, I would evaluate the effect of AVE3085 on reversal of pre-existing pressure overload-induced compensated cardiac dilation and decompensated dilation mice models, as this pathophysiology is more relevant to the patient clinical situation. In fact, AVE3085 has entered Phase I clinical trials for congestive heart failure in Europe (Sanofi Aventis business report 2007) and thus, has undergone pharmacokinetic and pharmacodynamic studies. The future study could therefore start at Phase II: according to the timeline of drug developments [24], if AVE3085 is approved by FDA, it may be launched to the market in a minimum of 7-10 years.

Current and future treatment of atherosclerosis

Atherosclerosis is a chronic inflammatory disease associated with dyslipidemia [25]. In addition to healthy lifestyle changes, current atherosclerosis therapies focus on lowering blood cholesterol levels, improving blood pressure control and preventing thrombotic complications [26]. However, these strategies are not effective in all patients, and do not directly address the inflammatory mechanisms driving atheroprogression [27, 28]. Results from the recent clinical trial “CANTOS” suggested that targeted anti-inflammatory therapy, i.e. canakinumab, a therapeutic monoclonal antibody targeting interleukin-1β, leads to reduced cardiovascular risk and atherosclerosis, without lowering cholesterol level [29]. However, effect on cardiovascular mortality was not observed in this trial, and patients who received canakinumab were at greater risk of death from infection than those who received a placebo [30]. The tremendous costs associated with this new compound, along with minimal health benefits, indicate that new therapeutic interventions able to decrease metabolic disorder-associated inflammation are required. Emerging evidence has strongly suggested that lymphatics are critical for immune response and cholesterol metabolism in atherosclerosis, indicating an essential contribution of lymphangiogenesis to atherosclerosis regression. Below, I will discuss prolymphangiogenesis as a potential novel therapeutic approach for atherosclerosis.

Therapeutic lymphangiogenesis – a new avenue for atherosclerosis treatment

The role of angiogenesis in atherosclerosis has been studied extensively; however, research on plaque lymphangiogenesis is relatively new. Lymphatics can be detected in human as well as mouse atherosclerotic plaque adventitia [31-33], suggesting a contribution to atherosclerotic lesion progression. Enhancement of lymphatic function represents a potential therapeutic target in atherosclerosis, through the promotion of immune cell (e.g. macrophage) egression from the plaque, and prevention of cholesterol accumulation. In addition, prolymphangiogetic therapy may prevent local edema, caused by leakage from the plaque vasa vasorum [32], or enhance the efficiency of proangiogenic therapy in CVD, by reducing revascularization and angiogenesis-related edema [34]. Advances in understanding the molecular underpinning of plaque lymphangiogenesis will offer insight into therapeutic options, potentially leading to the discovery of novel lymphangiogenic agents able to reverse atherosclerosis.

The identification of suitable target genes to influence the disease development is necessary for successful gene therapy [28]. In **chapter 6**, we provided a new insight in bioinformatics; demonstrating the success of a weighted gene coexpression network analysis (WGCNA) in identifying and validating key regulators of plaque lymphangiogenesis. Unlike a more traditional approach that focuses on differentially expressed individual genes, our approach is unique: we have employed WGCNA to capture high-order gene–gene interrelation in association with phenotypic trait i.e. plaque lymphangiogenesis. WGCNA gives information about functional connections, providing a true systems medicine perspective [35]. To provide proof of concept that WGCNA can identify functionally relevant genes, in **chapter 6**, we have investigated the role of the candidate genes, and demonstrated that TNIP2 is indeed a novel regulator of inflammatory lymphangiogenesis. Together, we provide insights that WGCNA in can effectively integrate cardiovascular gene expression and trait data, to identify novel therapeutic targets or potential genetic biomarkers of the diseases.

Perspectives on TNIP2 as a novel regulator of inflammatory lymphangiogenesis

We show in **chapter 6** that TNIP2 appears to be a specific regulator of pathologic lymphangiogenesis in atherosclerotic plaque instability. We postulate that TNIP2 may represent a much more suitable clinical target, possibly superior to other prolymphangiogenic agents. For instance, VEGF-C treatment has been reported to induce angiogenesis, increase inflammation and induce blood vascular leakage, leading to local edema which further impairs lymphatic function in experimental animal models [36, 37]. These deleterious effects may promote plaque instability and rupture. In contrast, TNIP2 is not a growth factor per se, but interacts with A20, which exerts anti-

atherogenic properties [38, 39]. TNIP2 did not affect angiogenesis in our zebrafish studies and possibly would not promote the blood vascular "side effects". Nevertheless, the role of TNIP2 in angiogenesis remains to be elucidated. The beneficial effects of TNIP2 may extend beyond its prolymphangiogenic properties, as TNIP2 can interfere with the inflammatory process in atherogenesis at multiple levels, i.e. NFκB, the Ang1/Tie2 axis, PPARs and IFNγ. It is well known that NFκB directly controls several proatherogenic genes and thus integrates multiple processes contributing to the formation of atherosclerotic plaques [40]. Indeed, TNIP2 exerts inhibitory effects on NFκB activation [41]. Moreover, it has been suggested that Ang 1/Tie2 exhibits anti-atherogenic effects [42]. Interestingly, TNIP2 structurally and functionally interacts with Ang1/ Tie2 signaling, leading to the inhibition of NFκB –dependent inflammatory gene expression [43-45]. Importantly, it has been suggested that anti-inflammatory drugs with pro-lymphangiogenic activity are appealing treat inflammatory conditions. This is because the activation of lymphatic function reduces the severity of tissue inflammation and thus, contributes to accelerated inflammation resolution [46, 47]. **In chapter 6**, we have highlighted such a regulatory role for TNIP2 in the PROX1 axis, enhancing PPAR-related lipid and cholesterol metabolism signaling and inhibiting that of IFNγ; indicating that TNIP2 exerts, not only lymphangiogenic, but also anti-inflammatory effects in lymphatic endothelial cells. Together, these findings suggest that TNIP2 may be an interesting target for the development of novel therapeutic strategies to treat inflammatory diseases, particularly in atherosclerosis. Further steps will have to be taken to confirm the findings in relevant animal models of atherosclerosis. For instance, target validation studies can be tested by overexpression or deletion of TNIP2 in atherogenic ApoE-deficient mice, or in mice with perivascular carotid collar placement-induced atherosclerosis. Subsequently, a small molecular weight chemical compound (SMOL) should be designed and tested *in vitro* and *in vivo*. In addition, the protection of intellectual property is required for the potential compounds, in order to eliminate the competition and to gain the freedom to operate [48]. Furthermore, large animal efficacy and toxicity studies are required; for which I prefer PCSK9-overexpressing mini pigs [49, 50], as their phenotype more closely resembles human plaques. Importantly, porcine models allow investigation of the impact of adventitial neovascularization by coronary imaging technologies [51, 52]. Thus, this may suitable to investigate adventitial lymphangiogenesis, and therefore will be easier to translate the results to the human situation. Subsequently, the suitable drug will eventually be utilized in multiple phases of human clinical trials and human randomized control studies. The particular target patients include those afflicted with atherosclerosis, MI and potentially lymphedema. In total, the *de novo* drug development requires 10-20 years before market launch [24] . GlaxoSmithKline may here be involved in developing the compounds; the company has an ongoing investigation of the TNIP2/ TPL-2/ NFκB1 p105 complex for anti-inflammatory pharmaceuticals [53].

In conclusion, although our findings cannot directly be translated into clinical application, we have provided a very promising concept which has therapeutic potential in future treatment and prevention of CVD. Further studies in combination with experimental verification are strongly encouraged to clarify their detailed role, and subsequently meet their clinical reality.

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