Summary and future perspectives

SUMMARY AND PERSPECTIVES

Cardiovascular disease is an umbrella term for any type of disorder that affects the heart and/or circulation. Despite improvements in knowledge and treatment options over the last decades, it remains one of the leading causes of disability and death. The underlying mechanisms vary depending on the disease in question. Most often there is not one cause for cardiovascular disease, but instead several risk factors are involved that increase the risk of development and progression of disease. Some of these risk factors can be avoided, controlled, treated or modified such as high cholesterol, high blood pressure and obesity. While others, such as family history and gender, cannot be avoided and their emphasis lies on monitoring and treatment. In this thesis, different mouse models were used to examine the role of DNA damage, atherosclerosis and the renin angiotensin system (RAS) on cardiovascular disease development and progression. Moreover, the present studies explored the effect of nutritional and therapeutic interventions.

This chapter describes the main findings of this thesis and concludes each part with suggestions for future research.

Part I Introduction

Chapter 2 provides an overview of the general structure and cell biology of the vessel wall. The vessel wall consists of three different layers termed tunica intima, tunica media and tunica adventitia. The main components of these layers include endothelial cells, vascular smooth muscle cells, cytoskeleton proteins and extracellular matrix proteins. Interaction between the components of these different layers determines the biological and physical properties of the blood vessel. Changes and damage to these components and cellular constituents contribute to the pathogenesis and progression of several vascular diseases, as well as to ageing of the vasculature.

In **Chapter 3**, the role of the RAS in the pathogenesis of vascular disease is reviewed. Activity of the RAS affects factors that contribute to the development and progression of vascular disease; i.e. extracellular matrix defects, atherosclerosis and ageing. Oxidative stress seems to be related to all of these components, subsequently contributing to the onset of vascular disease. Though, the precise mechanisms by which these components induce vascular damage is not entirely clear. RAS inhibiting therapies seem to have beneficial effects in treating cardiovascular disease, however, they are not 100 percent effective in all patients and occasionally even give rise to adverse side effects, including hypotension and hyperkalemia. Yet, when an angiotensin receptor blockade is used simultaneous with a neprilysin inhibition ('ARNI'), a much stronger favorable effect was found when compared to angiotensin receptor blockade only, without any extra associated negative side effects.[1, 2] However, insight into the mechanism of action of ARNI is still needed. Thus,

future research should explore optimal strategies of (combined) RAS blockade to prevent or stop the progression of vascular disease. Moreover, it would be particularly interesting to test the efficacy of combined RAS/reactive oxygen species suppressing therapy in animal models of cardiovascular disease, as it might give further beneficial effects on the vasculature.

Part II Aortic aneurysms

In part II, the effect of two risk factors, atherosclerosis and increased RAS signaling, on the development and progression of aortic aneurysms was studied. In **Chapter 4**, the relation between atherosclerosis and aortic aneurysms formation was investigated. In order to get a more representative physiological situation as observed in humans, aneurysmal susceptible heterozygous Fibulin-4 deficient mice (Fibulin-$4^{+/R}$) were combined with the atherosclerotic ApoE knockout mouse model. Our study showed that subtle thoracic aortic wall defects, such as increased elastic fiber fragmentation, induce increased atherosclerotic plaques formation and changes in plaque composition, including decreased elastin content, in ApoE$^{-/-}$Fibulin-$4^{+/R}$ mice after 10 weeks of high fat diet. Moreover, ApoE$^{-/-}$Fibulin-$4^{+/R}$ mice already showed relatively small thoracic dilatations when exposed to 20 weeks of low-fat diet. Interestingly, between 20 and 30 weeks of age, some of these ApoE$^{-/-}$Fibulin-$4^{+/R}$ mice developed symptoms of paralysis and 30% did not survive. These results indicate that subtle thoracic aortic wall defects in association with atherosclerosis, predisposes for development of aortic dilatations and altered plaque morphology. Whole body angiographs might help to further study the cause of paralysis and death observed in the ApoE$^{-/-}$Fibulin-$4^{+/R}$ mice. This will be a challenge since their death is quite sudden and unpredictable. Moreover, preliminary mouse RNA sequencing data suggests a difference in expression of genes involved in mitochondrial dysfunction and the immune system between atherosclerosis and aneurysm formation. Human RNA expression analysis similarly showed differences in immune pathway regulation between abdominal aneurysms and aortic occlusion (AAA vs AOD). Therefore, further analysis of specific immune factors in plasma of both mouse and human might shed light on the observed differences. It is evident that external factors such as diet have an enormous influence on aortic dilatations, especially abdominal. With this ApoE$^{-/-}$Fibulin-$4^{+/R}$ mouse model, we can now study the effect of high- and normal fat diet in a controlled manner, and elucidate the mechanisms that play a role in abdominal aneurysm formation and progression next to genetic factors.

Chapter 5 evaluated whether treating Fibulin-$4^{R/R}$ mice with the angiotensin II type 1 (AT_1) receptor antagonist losartan outperforms the effect of the renin inhibitor aliskiren or the effect of the β-blocker propranolol on aneurysm progression. Although both types of RAS blockers (losartan and aliskiren) identically lowered hemodynamic stress, only losartan increased survival, reduced aneurysm size and improved aortic wall distensibility. Moreover, losartan increased ejection fraction, decreased left ventricular diameter and

reduced cardiac TGF-β signaling, while the other drugs did not have these effects. None of the drugs examined here affected aortic wall morphology. To explain the beneficial effect of losartan compared to aliskiren, we reasoned that losartan offers an additional advantage, possibly by simulation of angiotensin II (AT_2) type receptors and/or activation of the angiotensin-(1-7)/Mas receptor axis. It still remains unclear whether RAS inhibition is effective in the prevention or reduction of aortic root dilations.[3, 4] Most clinical studies have a heterogeneous patient population in which they do not make a distinction between different underlying mutations, which makes it more difficult to draw firm conclusions. In addition, these clinical studies all start at different ages with their treatment, e.g. children, adolescent, adults and elderly. The strength of our study is the fact that these mice have the same genetic mutation, are treated from the same starting point, and accurate monitoring of aneurysm progression is followed within the same animal over time. Moreover, in this present study mice were treated postnatally, when aneurysm formation has already started, which is more clinically relevant as treatment of aneurysmal patients usually starts in the presence of an aneurysm. Timing of treatment as well as the underlying mutation are of utmost importance, as they may explain the success, or lack thereof, of different RAS blockers in clinical trials.[4, 6] Thus, future research should follow the same patient and/or animal over time, and should make a distinction between the underlying mutations of disease causing genes.

Part III Cardiovascular aging

In part III, the effect of defective DNA repair and the consequential aging process on the development of cardiovascular damage was examined. Previous studies have shown that the well-established premature aging *Ercc1*$^{d/-}$ mouse model shows signs of accelerated age-dependent vasodilator dysfunction, accompanied by increased blood pressure, vascular stiffness and vascular senescence. In **Chapter 6**, it was examined whether this accelerated age-dependent vasodilator dysfunction could be prevented by either treating the mice with the chronic AT_1 receptor blocker losartan, a well-known antihypertensive drug, or exposing them to dietary restriction, known to induce an anti-aging response. This study shows that dietary restriction is a very efficient intervention to prevent vasodilator dysfunction caused by genomic instability. Improvement of prostaglandin-mediated endothelium-dependent signaling and better vascular smooth muscle cell responses to nitric oxide were identified as mechanisms. Conversely, endothelial dysfunction was not reversible with chronic losartan treatment. These results suggest that this aging mouse model appears to represent the RAS blockade-resistant part of aging-related vascular disease. Accordingly, future research should use progeroid mouse models to further explore the underlying mechanism leading to age-related vascular disease and test the efficacy of drugs targeting vascular disease in order to extrapolate the results to the elderly population. A possible explanation for the lack of effect of losartan might be that vascular dys-

function in *Ercc1*$^{d/-}$ mice is largely ROS-independent, while often part of the detrimental effects of Ang II involve ROS formation. Additionally, *Ercc1*$^{d/-}$ mice show an upregulation of anti-oxidant and detoxification defense genes as part of a so-called survival response that aims to extend their lifespan.[7, 8] Research efforts should therefore also continue to fully elucidate the role of ROS in age-related vascular dysfunction.

In **Chapter 7**, the effect of aging on the heart was characterized and the use of fluorescent molecular markers for the early detection of cardiovascular disease was tested. MicroCT imaging showed that premature aging *Ercc1*$^{d/-}$ mice at 24 weeks of age display changes in left ventricular geometry and functioning, e.g. increased ventricular volumes and reduced ejection fraction. Results were similar when compared to functional analysis by echocardiography. Moreover, specific loss of *Ercc1* in cardiomyocytes, comparably showed adverse cardiac remodeling and poor cardiac functioning, suggesting the direct involvement of *Ercc1* in the heart. Furthermore, the combination of microCT and optical imaging allowed simultaneous analysis of molecular and functional changes in these mouse models for accelerated aging. Our study showed that a temporal increase in matrix metalloprotease activity and apoptosis precede cardiac functional decline in progeroid *Ercc1* mice. It would be interesting to investigate whether the increase in matrix metalloprotease activity is derived from senescent cells. In addition, this study did not investigate the direct causal effect of DNA damage on the development of heart failure; therefore, future research should be aimed at elucidating this question, for examples by measuring DNA damage markers in the hearts of the *Ercc1* mice, as well as inducing DNA damage by radiation and examining the effect on cardiac function. Furthermore, extensive analysis of mutations in DNA damage and repair genes leading to cardiovascular diseases is needed to expand our comprehension on how DNA damaging factors increase the susceptibility to cardiovascular event.

Part IV The renin-angiotensin system

In part IV, the role of the RAS was investigated. In **Chapter 8**, the use of the NIRF probe ReninSense680™ was tested to study renin activity *in vivo* and characterize renin activity in progeroid *Ercc1*$^{\Delta/-}$ mice which have premature age-related kidney pathology due to a defective nucleotide excision repair gene. Our study confirmed that *Ercc1*$^{d/-}$ kidneys display severe tubular attenuation and degeneration with marked anisokaryosis, which was shown previously.[9-11] In addition, we demonstrated that non-invasive imaging, using the NIRF probe ReninSense680™, enables imaging of altered renin activity in the kidney over time. The increased intrarenal activity detected with the ReninSense680™ probe after losartan treatment, is in full agreement with the literature, and thus not only validates the specificity of this probe, but also supports its use for longitudinal imaging of altered RAS signaling in aging. Moreover, our observations in the premature aging *Ercc1*$^{d/-}$ mouse model provides evidence that intrarenal renin activity does not necessarily run in parallel

with circulating renin. This observation was also seen in animal models of early diabetic nephropathy and patients with diabetes mellitus.[12-16] Hence, it would be interesting to further explore the association between aging and diabetes mellitus on the development of renal injury as well as the implications for future therapies; should we aim at locally lowering the intrarenal RAS in these models? Moreover, in several of the diabetic animal studies it was suggested that altered renin release from the kidney, and not reduced production, is responsible for the low circulating renin levels (reviewed within Price et al.)[14]. Future research should investigate the release and production of renin and other RAS components in aging mouse models.

In **Chapter 9**, our study re-evaluated the occurrence of (pro)renin in the brain, as the concept of a brain RAS has been controversial and this controversy continues to this day. It was found that buffer perfusion reduced mouse brain renin by approximately 60% and although renin-dependent Ang I-generating activity (AGA) could be detected in virtually every region, plasma renin was 40-800x higher than brain renin. Furthermore, deoxycorticosterone acetate (DOCA) salt, like Ang II, reduced circulating renin, and, contrary to our expectations did not increase brain prorenin. In fact, both DOCA-salt and Ang II lowered brain renin in parallel with plasma renin. Aliskiren-inhibitable AGA was entirely absent in the brain of Ren1c$^{-/-}$ mice, supporting the validity of our brain renin measurement. Thus, our data do not support the presence of locally synthesized, kidney-independent renin in the brain and we conclude that brain renin must represent renin that is taken up from blood. Moreover, the absence of Ang I in brain tissue outside the blood compartment strongly suggests that there is no local Ang I generation in the brain, and it appears that brain Ang II therefore originates from the blood compartment. Thus, it would be interesting to explore how this Ang II enters the blood-brain barrier, for instance by binding to brain angiotensin type-receptors that are outside the blood-brain barrier or rather by entering at sites where the blood-brain barrier permeability is compromised. In addition, the ReninSense probe holds considerable promise to localize and detect renin activity in tissues, and future studies should address the possibilities of assessing renin activity in other tissues than the kidney.

In **Chapter 10**, the use of VTP-27999, a novel renin inhibitor -without major side-effects at high doses- was examined in order to establish the maximum effect of renin inhibition in the kidney. The effect of VTP-27999 was compared to the clinically used renin-inhibitor aliskiren in 22 healthy volunteers on a low-sodium diet. A maximum effect of renin inhibition on renal plasma flow (RPF) and glomerular filtration rate (GFR) was found at VTP-27999 doses of 300 mg and higher, while a maximum effect was not reached with aliskiren at a dose of 300 mg. The maximum effect of VTP-27999 was approximately 30% higher than the effect of aliskiren at the same dose. The maximum effect of VTP-27999 can most likely be reached with 600 mg of aliskiren, however such high doses of aliskiren are clinically not recommended because of its side effects. With this study,

we could establish that maximum RPF increases cannot be established with aliskiren at clinically relevant doses. As our study only included healthy patients, future research in patients with diabetes, hypertension and/or kidney disease is necessary to confirm these results, as the use of RAS blockers increases the risk of adverse events, including hyperkalemia, in this population. In addition, it would be interesting to test the effect of VTP-27999 in an aging mouse model, as they may have an altered RAS activity resulting in a different responsiveness. Moreover, in this study we did not compare males and females, thus further research should distinguish between males and females to determine whether or not the effect is similar in both genders.

REFERENCES

11. Tummala R, Bhadra R, Gupta A, Ghosh RK. Combined neprilysin and ras inhibition in cardiovascular diseases: A review of clinical studies. J Cardiovasc Pharmacol. 2016;68:183-190
12. Uijl E, Roksnoer LC, Hoorn EJ, Danser AH. From arb to arni in cardiovascular control. Curr Hypertens Rep. 2016;18:86
13. Brooke BS, Habashi JP, Judge DP, Patel N, Loeys B, Dietz HC, 3rd. Angiotensin ii blockade and aortic-root dilation in marfan's syndrome. N Engl J Med. 2008;358:2787-2795
14. Lacro RV, Dietz HC, Sleeper LA, Yetman AT, Bradley TJ, Colan SD, Pearson GD, Selamet Tierney ES, Levine JC, Atz AM, Benson DW, Braverman AC, Chen S, De Backer J, Gelb BD, Grossfeld PD, Klein GL, Lai WW, Liou A, Loeys BL, Markham LW, Olson AK, Paridon SM, Pemberton VL, Pierpont ME, Pyeritz RE, Radojewski E, Roman MJ, Sharkey AM, Stylianou MP, Wechsler SB, Young LT, Mahony L, Pediatric Heart Network I. Atenolol versus losartan in children and young adults with marfan's syndrome. N Engl J Med. 2014;371:2061-2071
15. Dietz HC. Potential phenotype-genotype correlation in marfan syndrome: When less is more? Circ Cardiovasc Genet. 2015;8:256-260
16. Groenink M, den Hartog AW, Franken R, Radonic T, de Waard V, Timmermans J, Scholte AJ, van den Berg MP, Spijkerboer AM, Marquering HA, Zwinderman AH, Mulder BJ. Losartan reduces aortic dilatation rate in adults with marfan syndrome: A randomized controlled trial. Eur Heart J. 2013;34:3491-3500
17. Niedernhofer LJ, Garinis GA, Raams A, Lalai AS, Robinson AR, Appeldoorn E, Odijk H, Oostendorp R, Ahmad A, van Leeuwen W, Theil AF, Vermeulen W, van der Horst GT, Meinecke P, Kleijer WJ, Vijg J, Jaspers NG, Hoeijmakers JH. A new progeroid syndrome reveals that genotoxic stress suppresses the somatotroph axis. Nature. 2006;444:1038-1043
18. Schumacher B, van der Pluijm I, Moorhouse MJ, Kosteas T, Robinson AR, Suh Y, Breit TM, van Steeg H, Niedernhofer LJ, van Ijcken W, Bartke A, Spindler SR, Hoeijmakers JH, van der Horst GT, Garinis GA. Delayed and accelerated aging share common longevity assurance mechanisms. PLoS Genet. 2008;4:e1000161
19. Dolle ME, Kuiper RV, Roodbergen M, Robinson J, de Vlugt S, Wijnhoven SW, Beems RB, de la Fonteyne L, de With P, van der Pluijm I, Niedernhofer LJ, Hasty P, Vijg J, Hoeijmakers JH, van Steeg H. Broad segmental progeroid changes in short-lived ercc1(-/delta7) mice. Pathobiol Aging Age Relat Dis. 2011;1

20. Schermer B, Bartels V, Frommolt P, Habermann B, Braun F, Schultze JL, Roodbergen M, Hoeijmakers JH, Schumacher B, Nurnberg P, Dolle ME, Benzing T, Muller RU, Kurschat CE. Transcriptional profiling reveals progeroid ercc1(-/delta) mice as a model system for glomerular aging. BMC Genomics. 2013;14:559
21. Vermeij WP, Dolle ME, Reiling E, Jaarsma D, Payan-Gomez C, Bombardieri CR, Wu H, Roks AJ, Botter SM, van der Eerden BC, Youssef SA, Kuiper RV, Nagarajah B, van Oostrom CT, Brandt RM, Barnhoorn S, Imholz S, Pennings JL, de Bruin A, Gyenis A, Pothof J, Vijg J, van Steeg H, Hoeijmakers JH. Restricted diet delays accelerated ageing and genomic stress in DNA-repair-deficient mice. Nature. 2016;537:427-431
22. Hollenberg NK, Fisher ND, Nussberger J, Moukarbel GV, Barkoudah E, Danser AH. Renal responses to three types of renin-angiotensin system blockers in patients with diabetes mellitus on a high-salt diet: A need for higher doses in diabetic patients? J Hypertens. 2011;29:2454-2461
23. Zimpelmann J, Kumar D, Levine DZ, Wehbi G, Imig JD, Navar LG, Burns KD. Early diabetes mellitus stimulates proximal tubule renin mrna expression in the rat. Kidney Int. 2000;58:2320-2330
24. Price DA, Porter LE, Gordon M, Fisher ND, De'Oliveira JM, Laffel LM, Passan DR, Williams GH, Hollenberg NK. The paradox of the low-renin state in diabetic nephropathy. J Am Soc Nephrol. 1999;10:2382-2391
25. Jaffa AA, Chai KX, Chao J, Chao L, Mayfield RK. Effects of diabetes and insulin on expression of kallikrein and renin genes in the kidney. Kidney Int. 1992;41:789-795
26. Correa-Rotter R, Hostetter TH, Rosenberg ME. Renin and angiotensinogen gene expression in experimental diabetes mellitus. Kidney Int. 1992;41:796-804