Rare genetic variants associated with early onset CVD
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Chapter 10

Summary and Future perspective
Summary

The main aim of this thesis project was to unravel the molecular basis of early onset CVD in families. The heritability contributes to a large extent to the disease phenotype. Additionally we were interested in the identification of biomarkers that could help to predict early onset CVD.

In Part I of the thesis I investigated whether circulating monocyte gene expression isolated from blood of patients can be used as novel biomarkers explaining the disease phenotype. In part II I report on our approach to identify the causal genetic variants in families with premature CVD.

Chapter two is a systematic overview summarizing the current knowledge on the use of gene expression in circulating monocytes as putative biological marker to identify individuals at risk for CVD. We searched the PUBMED databases for publication on CVD and monocyte gene expression. By comparing selected studies we came to the conclusion that the methods for the isolation of circulating monocytes is crucial for the outcome. It became very clear that there is insufficient evidence that gene expression of circulating monocyte can be applied as a diagnostic tool. We observed an inconsistency in gene expression profiles in the different studies, which could be due to the relative small sample sizes, variation in isolation methods and in clinical phenotype of the included patients as well as the influence of medication use.

In chapter three we presented a case-control study on the gene expression level of circulating monocytes in patients with premature atherosclerosis versus healthy controls in the presence or absence of treatment with statins. A total of three genes, \textit{ABCA1}, \textit{RGS1} and \textit{ADRB2} were differentially expressed and validated in a second case-control cohort. The expression of \textit{ABCA1} and \textit{RGS1} was decreased whereas \textit{ADRB2} expression was increased in the patients versus controls. However, the observed fold changes were small and should be interpreted carefully. For \textit{ABCA1} and \textit{ADRB2} a possible association with CVD is known. \textit{ABCA1} participates in the removal of cholesterol from lipid-laden macrophages to premature HDL particles. Common polymorphisms in \textit{ADRB2} have been shown to be associated with decreased CVD risk. A direct association of \textit{RGS1} on the atherogenesis of CVD is still unclear.

In chapter four a study is presented to analyze the circulating monocyte transcriptome of healthy volunteers who received a LPS challenge to stimulate the immune system. The exposure to endotoxin resulted in the up- or downregulation of several genes linked to systemic inflammation in the context of atherosclerosis. In total six genes were identified: \textit{BATF}, \textit{BID}, \textit{C3aR1}, \textit{IL1RN}, \textit{SEC61B} and \textit{SLC43A3}. Importantly, this study underlines the feasibility of using a pro-inflammatory challenge to evaluate the inflammatory response of the major immune
cells involved in atherosclerosis: the monocytes. Theoretically, this technique can be implemented in future studies to identify patients/families with a hyper-responsive, pro-inflammatory phenotype, which could contribute to accelerated atherogenesis.

In chapter five I present a study to investigate if in a minority of patients suffering from an acute myocardial infarction (AMI) without detectable lesions upon coronary angiography (Myocardial Infarction Normal Coronary Arteries (MINCA)) a marker to diagnose these patients can be elucidated. We investigated whether coagulation parameters were associated with MINCA. Carotid Intima Media Thickness (cIMT), plasma lipid levels, hemostatic parameters and in-vitro thrombin generation were measured. cIMT was similar in MINCA patients and significantly decreased compared to patients with AMI. Plasma lipid levels did not differ between the groups. Tissue plasminogen activator (tPA) and plasminogen activator inhibitor-1 (PAI-1) were significantly increased in both patient cohorts compared to healthy controls. The ex-vivo thrombin generation parameters (ETP and peak) were significantly increased in both cohorts compared to healthy controls in the presence of 1 pM Tissue Factor. Thus, both MINCA and AMI patient were characterized by a proatherothrombotic state compared to healthy controls. The elevated levels of tPA and PAI-1 in MINCA does reflect a change in the fibrinolytic system. However, we were unable to distinguish the MINCA phenotype from patients with CVD. Based on our study it is evident that MINCA patients should be treated to restore the coagulation abnormalities.

In chapter six, I present a review summarizing published data from GWAS studies pointing to novel genes involved in the pathophysiology of atherosclerosis. GWA studies identify the ‘common disease, common variant’ hypothesis in which a limited number of genetic variants with a high frequency contribute to a disease. Although 17 loci have been identified to be associated with CVD only part of the heritability is currently explained. A substantial part will be explained by rare variants with severe effects on the phenotype. Therefore combining family studies and NGS could help to reveal those variants.

In Chapter Seven we report on the study to elucidate the genetic background of a large multigeneration family with 11 members suffering from early onset CVD by using classical linkage analysis. We identified a 4.4 Mb Interval on chromosome 12 that was linked to the phenotype with a parametric LOD-score of 3.31. The subsequent capture and sequencing of this region resulted in the identification of one non-synonymous variant in Keratocan (KERA; NM_007035.3: c.920 C>G; p.Ser307Cys), encoding for an extracellular protein. The variant was not present in 10,386 healthy controls or in 1,962 patients with premature CVD. Keratocan was
not present in healthy artery segments, but heavily abundant within the lipid core of atherosclerotic lesions. In Apoe\(^{-/-}\) mice, wherein atherosclerosis was induced by cuff placement in the vessel wall, a highly significant linear association was found between Keratocan expression and plaque size \((r^2 = 0.69, p < 0.0001)\). Based on these data we concluded that KERA might be a novel player in the pathological process underlying atherosclerosis. Yet additional studies are required to elucidate its exact role.

**In Chapter eight** a study is presented to identify the mutation responsible for the early premature atherosclerosis phenotype in a small family with multiple individuals with early onset CVD. Due to the smaller size of the family we performed exclusion linkage analysis in combination with exome sequencing. We identified three variants co-segregating with the disease phenotype in the family CAMPSAP1, ZC3HC1 and MCF2L. The CAMPSAP1 variant was found with a high frequency in healthy controls and was eliminated from further studies. The ZC3HC1 variant was detected in five additional PAS cases but no co-segregation with the phenotype could be demonstrated and thus the variant was eliminated from further studies. The MCF2L variant (encoding DBS, c.2006 A > G; p.Asp689Gly) was the only promising variant. We showed that MCF2L-Asp689Gly was defective in activating RAC1 and did not induce stress fibers in transfected HeLa cells indicating a deficiency providing the shift of GDP to GTP in GTPases and their downstream pathway targets. These defects may result in increased permeability and perturbed cell migration. Previous studies showed that GEFs together with GTPases play a pivotal role in leukocyte migration and therefore in the context of CVD. A limitation of the study is the fact that we cannot rule out that due to the used filtering approach of the genetic data, other rare variants might have been filtered.

Finally, in **Chapter Nine** I summarize the method I have used to successfully reprogram patient-fibroblasts into induced pluripotent stem cells (iPSCs). This technique could be very valuable as patient-specific stem cells can be produced carrying the specific variants and haplotypes of the subject. They can be used for disease modelling by using their potential to differentiate into cell types involved in the disease pathobiology and drug design. Additionally, the correction of a genetic defect could be used to validate proof-of-concept for cell therapeutic applications. Furthermore ‘healthy’ iPSCs could be used for tissue repair and cell production such as red blood cells or platelets for transfusion medicine. iPSC can help to understand the complex pathophysiology of CVD.
Future perspective

Cardiovascular disease (CVD) is the major cause of morbidity and mortality in Western Societies. It has been the subject of an immense amount of basic and applied research to understand its complex molecular pathobiology. A family history of premature CVD is a known independent risk factor for cardiovascular events. However, the exact contribution of genetic variants to the disease phenotype remains to be elucidated. Based on current knowledge, derived from GWAS studies, it was reported that common variants explain approximately 10% of the disease risk [1]. On the other hand, rare variants with a large effect size, identified in families with Mendelian forms of CVD, may explain to a large extent the disease phenotype [2-7]. So far linkage studies in large pedigrees have only identified a few novel causal variants (LRP6, MEF2A) on top of the already known loss of function mutations in the LDLR. By using next generation sequencing (NGS) and linkage analysis we successfully analysed the genetic background of two families with premature atherosclerosis, presented in this thesis, identifying variants in KERA and MCF2L as likely pathogenic. Yet, the fact that these 2 candidates could only be identified in the most promising families, after a long scientific journey, it is highly unlikely that GWAS will become a part of routine diagnostic procedures in an outpatient setting for these patients. Conversely, the fact that these candidate genes were found in PAS families does imply that these genes may point towards potentially attractive intervention pathways to further reduce the residual CVD burden.

Based on the current knowledge it is anticipated that broader strategies than merely GWAS have to be used to identify novel causal genetic variants. In this context whole genome sequencing in combination with exome sequencing, transcriptome microarray analysis of different cells isolated from patients with extremely well-defined disease phenotype, RNA sequencing will all be instrumental to fuel the pipeline of system biology analysis to understand the molecular mechanisms and signalling pathways underlying the disease of interest. It is an emerging field, for example, recently, long non-coding RNAs and small non-coding RNAs, so called miRNAs, have been shown to play an important role in gene regulation. Only one-fifth of the transcriptome in the human genome is associated with protein-coding genes and as of June 2014, 197 long non-coding RNAs have been functionally annotated. Non-coding RNAs modulate the function of transcription factors or control the activity of co-regulators. More than 2500 miRNAs are currently identified in the human genome (http://www.mirbase.org/) and the most relevant miRNAs related to CVD are ANRIL, CDKN2A, CDKN2B and MTAP in the 9p21 locus [8]. Of note, with so many unknown factors in such a heterogeneous disease, it is also imperative to greatly improve detailed disease phenotyping, allowing for intelligent clustering of specific
subtypes of disease. For instance, with the advent of PET/CT monitoring as a means to identify patients with increased inflammatory activity in the atherosclerotic lesions, combined with extensive animal data supporting the detrimental impact of increased inflammation in atherosclerotic plaques, it would greatly facilitate target discovery if such techniques would be routinely used to perform in-depth phenotyping in families with unexplained PAS.

Furthermore the impact of epigenetics is now emerging as an important key player in the CVD pathophysiology [9]. Epigenetics refer to changes in the modifications of histones and/or DNA (methylation and acetylation), which greatly affect the availability of DNA for transcribing RNA. It has been shown that DNA methylation inhibitors, histone methylation inhibitors and histone deacetylase inhibitors have been successfully used in cancer treatment [10]. In view of the close relation between epigenetic alterations and the hyperresponsiveness of e.g. immune cells [9,11], epigenetic alterations can be expected to also associated with hyperinflammatory reactions in atherosclerosis.

System biology is needed to model all interactions between genomics, transcriptomics, proteomics and epigenetics, all in relation to the ‘detailed’ phenotyping studies of the individual patients/families. In this context the use of patient-specific iPSCs and/or gene corrected iPSCs and their differentiation into disease related cells, cell signalling networks could be a promising tool.

Even though current treatment to prevent CVD has been very successful, the majority of cardiovascular events are not prevented. Results from cell biology- and animal studies will need to be integrated carefully to choose the next generation of therapeutic targets. An improved understanding of the molecular mechanism and useful biological markers to identify personal risk of individuals could lead to novel targets for therapy and prevention.
References


Summary and Future Perspective

