Identification and targeting of genes in atherosclerosis
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Chapter 13

Summary, General Discussion and Future Perspectives

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Chapter 13
SUMMARY

The aim of the studies described in this thesis was to assess the relevance of variations in known and novel genes for CVD associated endpoints in order to ultimately identify therapeutic targets for the treatment of atherosclerosis. The type of research is truly translational; it nourishes on the data derived from our outpatient clinics and hospital wards. Despite the vast arsenal of drugs and interventions currently available, residual cardiovascular disease risk remains high. We focused on genetics as a tool for the identification of new targets for therapies. Cardiovascular disease and cholesterol levels are both driven for a large part by genetic factors, of which a majority is still unknown. In analogy to the effective LDL-C lowering therapies, once initiated on the basis of epidemiologic and genetic studies, HDL-C and triglycerides (TG) have emerged as new therapeutic targets. In part I, we studied new genetic variants in relation to HDL and TG levels in families. Next, we moved to therapeutic targets currently in development that are derived from previous genetic studies in part II. Finally, in part III we discuss new potential targets in unknown pathways, identified in rare families with an autosomal pattern of cardiovascular disease in the absence of traditional risk factors.

In Part I we focused on the identification of new genetic variants associated with HDL-C and triglyceride levels. These studies were largely conducted in families that were referred to our outpatient clinic for the analysis of the origin of an observed autosomal dominant form of extreme HDL-C or triglyceride levels.

In Chapter 2 we studied the effects of genetic variation in LRP1 on HDL metabolism. LRP1 is generally considered a modulator of triglyceride rich lipoprotein metabolism and is associated with both HDL-C and triglyceride levels in GWA studies. In addition, an LRP1 variant was shown to be associated with apoA-I levels, but not with apoB levels in the Copenhagen Heart study. In these large-scale studies, common (intronic) variants with a small effect on the clinical endpoint are investigated. We, however, focused on the clinical consequences of rare exonic variations that were found in families with extreme forms of dyslipidemia. These two rare LRP1 variants were associated with low HDL-C and moderately high triglyceride levels. In vitro analysis of the rare variants indicated that loss-of-function of LRP1 resulted in a lower cell surface expression of ABCA1 and SR-B1, two transcellular proteins with a known effect in HDL metabolism, suggesting that LRP1 influences HDL metabolism in humans directly; the observed low HDL-C phenotype associated with LRP1 mutations is therefore considered not only to result from alterations in TG metabolism.

Chapter 3 describes several families with rare variants in ABCA8, which was previously identified as a locus associated with HDL-C levels in GWAS. The mechanism underlying the observed association is not known and the fact that we identified mutations that were
Chapter 13

considered to have an effect on ABCA8 function allowed for studies to address the role of ABCA8 in human lipid metabolism. The ABCA8 variants in the families were associated with low HDL-C levels and in additional studies we showed that ABCA8b knockout mice display lower HDL-C levels. As ABCA8 is part of the same protein family as the classical HDL protein ABCA1, we hypothesized there could be similarities in the function of these proteins. ABCA1 and ABCA8 colocalized at the cellular membrane and have a similar intracellular localization pattern. In functional tests, we showed that ABCA8 has a role in transmembrane efflux of cholesterol to apoA-I, but this effect was not as potent as the effect observed for ABCA1. The exact mechanism how ABCA8 induces cholesterol efflux is not known and this warrants for further studies. ABCA8 could potentially be pivotal in the transports of lipids to specific membrane domains, hereby contributing to the lipid composition of these membrane domains and creating regions from which ABCA1 can then transport lipids to ApoA-I.

In Chapter 4 we switched from HDL to triglyceride metabolism and reported two new loss-of-function variants in APOC3. The APOC3 variants are associated with low apoC-III protein levels, high HDL and low triglyceride rich lipoprotein levels. Two recent Mendelian randomization studies confirm our findings and clearly show that genetic loss-of function variants in APOC3 are indeed associated with a favorable lipid profile and, most importantly, to a reduction in coronary artery disease (CAD) events and overall mortality.

In Part II we focused on new therapeutic targets for the treatment of cardiovascular disease that influence HDL, triglyceride and Lp(a) metabolism. In Chapter 5 and 6 we continued our studies on apoC-III, now established as an important therapeutic target based on the abovementioned genetic studies. In the EPIC-Norfolk prospective population study, 2711 individuals were studied of whom 832 developed CAD during follow-up.

In Chapter 5, we measured apoC-III plasma levels in baseline samples and established that apoC-III levels predict CAD risk, independent from cardiovascular risk factors. ApoC-III is a key regulator of triglyceride rich lipoproteins and a determinant of many lipoprotein levels. We next questioned which of these processes mediate the association on coronary artery disease risk and found that elevated levels of remnant lipoproteins, small dense LDL and low-grade inflammation largely explained this association. Taken together, this study provides further support for apoC-III as a therapeutic target.

One of the major, unresolved issues regarding apoC-III metabolism is the distribution of apoC-III among circulating lipoproteins. Several studies have identified specific subgroups of subjects at increased CAD risk based on the number of apoC-III containing LDL or HDL particles, but their association with CAD risk has been inconsistent. In Chapter 6 we measured lipoprotein associated apoC-III levels in the same EPIC-Norfolk cohort, using a new ELISA based method that enables high throughput analysis in large sample sizes. The apoCIII-apoB and apoCIII-apoAI measures reflect the amount of apoC-III on saturating levels of apoB-100
General Discussion

and apoA-I, which is a measure of the average apoC-III content per lipoprotein. The indices of ‘total apoCIII-apoB’ and ‘total apoCIII-apoAI’ are derived by multiplying these measures with plasma apoB and apoA-I levels and reflect the total apoC-III content in the respective lipoprotein pools. apoCIII-ApoAI and apoCIII-apoB were not associated with CAD risk in multivariable adjusted analysis, and did not provide additional value over total plasma apoC-III. The index of ‘total apoCIII-apoB’, but not ‘total apoCIII-apoAI’ was predictive for CAD risk, which is in line with previous reports. Finally, we identified Lp(a) associated apoC-III as a significant predictor of CAD risk after adjustment of other risk factors, and a potential new CAD risk marker.

Chapters 7 and 8 studied HDL and in Chapter 7 the key question was whether apoA-I, the main apolipoprotein in HDL is a better predictor of CAD risk compared to HDL-C. This issue has become relevant as several therapies that increase HDL-C did not translate to clinical benefit. A potential explanation could be that not the cholesterol content of HDL (HDL-C) per se should be targeted, but rather the functionality of HDL. One of the suggested parameters that might be a better representation of HDL’s function is apoA-I. However, in three large independent prospective studies we showed that apoA-I levels did not offer predictive value over HDL-C levels. In fact, in several quartiles there was even an association with increased cardiovascular risk, which can be related to increased levels of cardiovascular risk factors that were associated with apoA-I levels.

In Chapter 8 we studied the effects of the CETP inhibitor, TA-8995, on a measure of HDL functionality: cholesterol efflux capacity (CEC). Previous studies have shown that CEC is a strong predictor of cardiovascular risk, independent from HDL-C. Here we showed in a randomized controlled trial that treatment with TA-8995 resulted in potent increases in total, as well as ABCA1 and non-ABCA1 driven CEC. Whether increasing CEC results in cardiovascular disease reduction has never been demonstrated to date and requires formal testing in a cardiovascular outcome trial. TA-8995 is one of the few drugs that can be used for such a study.

As the last chapter of this part, we move on to Lp(a) as a new target for therapy in Chapter 9. Elevated Lp(a) levels have long since been identified as a cardiovascular risk factor and recent genetic studies have provided clear support for a causal association. Progress in Lp(a) research has severely been hampered by a lack of effective strategies to lower Lp(a). Here, we report the effect of apo(a) antisense therapy with IONIS APO(a)Rx in 64 patients and APO(a)-L*_Rx, the updated, more potent ligand conjugated antisense (LICA, hence L*_Rx) compound, in 58 volunteers. Treatment resulted in sustained Lp(a) reductions of ±70% for APO(a)_Rx and >90% for APO(a)-L*_Rx. Antisense therapy was generally well tolerated and especially the new APO(a)-L*_Rx compound was associated with a low number of reported side-effects. Sustained Lp(a) reductions >90% were unprecedented prior to this study and this therapy finally provides the tools for a cardiovascular outcome trial to test the promise of Lp(a) as a therapeutic target. One of the first clues was already provided. The atherogen-
ic effect of Lp(a) is thought to be related to the content of oxidized phospholipids which has strong pro-inflammatory effects on monocytes and endothelium. We showed that Lp(a) lowering resulted in a significant reduction of the inflammatory profile of monocytes, a process that is reverted after Lp(a) levels return to baseline in the washout phase.

Finally, in Part III we studied families with an observed autosomal dominant form of early cardiovascular disease, in the absence of traditional cardiovascular risk factors. In Chapter 10 we described a family comprising 4 generations of whom 11 members suffered from early cardiovascular disease. Using traditional linkage analysis, we observed a 4.4Mb interval on chromosome 12 with a parametric LOD-score of 3.31. Upon sequencing, we identified a rare non-synonymous variant in KERA. KERA codes for keratocan (KERA), an extracellular proteoglycan with an unknown role in atherosclerosis. Interestingly, KERA was not expressed in human healthy arterial wall segments, but was found in the vicinity of atherosclerotic plaque regions. In a follow-up study in mice, we found that the extent of atherosclerotic lesion formation was significantly associated with KERA protein expression over time.

In a different, smaller family we identified a rare variant in MCF2L using a combination of exclusion linkage analysis and whole exome sequencing, which is reported in Chapter 11. The protein was not found in healthy arterial wall segments but was found in diseased human atherosclerotic plaque segments. MCF2L is a guanine exchange factor that interacts with Rho/Rac proteins and is involved in cellular signaling pathways. We showed in in-vitro studies that the identified variant was defective in activating Rac1 and intracellular actin stress fibers failed to develop, indicating this variant results in impaired Rac1 activation. Based on these findings potential mechanisms of action are a role in endothelial function and / or regulation of leucocyte migration. Follow up studies are needed to further understand the role of MCF2L in atherosclerosis and confirm our findings.

In Chapter 12 we report the ongoing work on a third family with early-onset cardiovascular disease. In this family, we used whole exome sequencing to identify a non-sense variant in SUSD2, coding for sushi domain containing protein 2. Further support for an association with cardiovascular disease is derived from GWA studies. In CARDioGRAMplusC4D common variants in SUSD2 were associated with cardiovascular disease after Bonferroni correction for the number of tested SNPs. SUSD2 is expressed in the arterial wall and specifically in pericytes, regulatory cells, wrapped around endothelial cells. Additional studies investigating the role of SUSD2 in atherosclerosis and endothelial homeostasis are ongoing.
General Discussion and Future Perspectives

Current therapeutic targets in development: HDL vs TRL

In retrospect, it is worthwhile to look back on our review on HDL targeted therapies in 2013 and reflect on the optimistic nature of the review. In the last 4 years, a number of clinical trials have been conducted and they all failed to show a beneficial on (surrogate) cardiovascular endpoints. The start of this PhD project coincided with the publication of the hallmark Mendelian randomization paper by Voight et al, who applied a Mendelian randomization design to test whether HDL-C and TG levels are causally related with incident CAD outcome measures. Genetic association studies have consistently shown a direct and causal role of triglyceride (rich lipoproteins) in atherosclerosis development. Two important clinical targets stand out: apoC-III and ANGPTL3 for which antisense therapies are currently in advanced stages of development. Clinical outcome studies are eagerly awaited to evaluate the clinical potential of these new compounds. Coming back to HDL genetics, it is often overlooked in the initial Mendelian randomization study that there was one HDL locus that was associated with reduced CAD risk: CETP, an association that was recently confirmed in a large genetic study. Recently, Merck announced that the REVEAL (Randomized EValuation of the Effects of Anacetrapib through Lipid modification) outcomes study of the CETP inhibitor anacetrapib met its primary endpoint, significantly reducing major coronary events. As argued in previous chapters of this thesis, CETP inhibitors both increase HDL-C and decrease LDL-C levels and therefore do not answer the validity of HDL-C as a therapeutic target. In fact, only one locus is associated with CAD risk and HDL-C levels, without affecting other lipoproteins: SCARB1 coding for the scavenger receptor class BI (SR-BI), a major HDL receptor. In this study individuals with a loss-of-function mutation in SR-BI displayed higher HDL-C levels, and increased CAD risk, possibly due to impaired reverse cholesterol transport. This study provides the first evidence in support of a causal association between HDL and cardiovascular risk and for it to be in the opposite direction as expected from epidemiological studies is ironic, to say the least. This indicates that HDL-C is not a static measure but reflects the net result of the dynamic process of HDL synthesis, remodeling and catabolism/uptake. It clearly highlights the need for a better functional understanding of HDL-metabolism in humans as we have pursued in part I of this thesis.

The old kid on the block: Lp(a)

Although it has first been identified as early as the 50’s, no verdict on the effect on cardiovascular outcome is available, largely due to a lack of selective drugs that lower Lp(a) effectively. The combination of the available genetic studies and our work on apo(a) antisense in chapter 10, build a case for a large outcome trial. Currently a phase II study for the
Chapter 13

updated LICA antisense compound is recruiting (NCT03070782) and a subsequent cardiovascular outcome study is eagerly anticipated.

Identifying new (non-lipid) targets for the treatment of atherosclerosis

The availability of statins, ezetimibe and PCSK9 inhibitors enable us to adequately lower LDL-C levels in virtually all patients. Despite these efforts residual CAD risk remains high. For instance, in a recent study recurrent CAD risk was still 12.6% after 3 years follow-up, despite median on-treatment LDL-C levels as low as 0.78 mmol/L.17 An explanation for this large residual risk could very well be related to other lipoprotein risk factors at play like triglyceride rich lipoproteins and Lp(a), independent from LDL-C. As argued in the previous paragraph there are therapies in advanced stages (apoC-III, ANGPTL3, Lp(a) ) of development to test this hypothesis. In parallel, efforts are undertaken to identify additional targets for therapy to enable further CVD risk lowering. In light of this, it is important to emphasize that these therapies should target the current pivotal processes in disease progression. Especially since there is a transition in clinical presentation of CAD events observed in the past decade: from myocardial infarction with ST elevation (STEMI) to a majority presenting with non-ST elevated myocardial infarction (NSTEMI) and a corresponding shift in atherosclerotic plaque morphology is observed.18 This development is suggested to be related to a better identification and treatment of CAD risk factors, notably through LDL-C lowering by statins. Taken together, these findings call for a critical reassessment of the underlying pathophysiology in CAD development, beyond LDL-C. Data derived from large genetic association studies might provide these new targets in the future, but to date, no target has been reached in clinic yet, and as such, results on the new CAD associated loci are disappointing. Most of the new variants identified in GWAS are located in regions of genes with an established role in CAD, which can be regarded as a proof that GWAS is indeed a valid tool for target identification.19 The role of the majority of the variants identified, however, is not known, which requires extensive additional studies.20 As an alternative strategy we propose to study families with a heritable pattern of severe atherosclerosis in the absence of cardiovascular risk factors, especially LDL-C, as we do in Part 3 of this thesis. Such families with normal LDL-C may very well be a better representation of the average CAD patient in 2017 than the ‘old’ CAD cases that were enrolled in the large association studies originating from the ‘90s. Another option would be to perform association studies in cases preselected on low LDL-C levels to identify alternative pathways that contribute to atherosclerosis.

It is interesting to note that several new CAD associated loci are highly expressed in vasculature/endothelial cells12 which is in line with some of our own data in part III of this thesis. Phenotype association studies also point to the role of endothelium in the development of atherosclerosis.21,22 It certainly fits with the increasing appreciation of the active contribution of the endothelium to leucocyte infiltration and plaque development. Biochemical
techniques progress and iPSC and Blood Outgrowth Endothelial Cells (BOEC) can provide us patient-derived endothelial cells that can be used in 3D flow systems that mimic the human vasculature.\textsuperscript{23} In a future world where any combination of lipoprotein abnormalities can be treated with statin/PCSK9, apoC-III/ANGPTL3 or Lp(a) based therapies, this might very well be a fruitful area for the discovery of new non-lipid related treatment targets.
Chapter 13

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