The Clinical Value of HDL Function Measurements

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CHAPTER 6

General discussion

This thesis evaluated the potential clinical usefulness of assessing HDL function in the context of cardiovascular disease (CVD) using both cross-sectional and prospective approaches. CVD is the leading cause of death worldwide [1]. CVD comprises diseases of the heart and blood vessels, including coronary heart disease (CHD), peripheral arterial disease and cerebrovascular disease [2]. Atherosclerosis is the main underlying pathology in CVD development and dyslipidemia is an important risk factor for atherosclerosis [3]. Lipid abnormalities, including high levels of low-density lipoprotein cholesterol (LDL-C) and low levels of high-density lipoprotein cholesterol (HDL-C), are associated with an increased risk of CV events. Interventions to reduce LDL-C have been successfully developed [4,5]. However, these interventions often fail to reach the desired LDL-C concentrations, leading to a remaining risk for CVD [6-8]. It is therefore essential to discover alternative approaches and to identify targets suitable for further CVD risk reduction. Increasing HDL-C concentrations was considered as such an alternative target to reduce CVD risk, as many prospective and epidemiological studies have confirmed that HDL-C is an independent predictor of incident cardiovascular events [9-11]. However, trials which specifically targeted raising HDL-C levels failed to reduce CVD risk [12-14] .In addition, genetic studies demonstrate that life-long high or low HDL-C levels in the general population do not translate into the expected reduced or increased risk of CVD, respectively [15]. Combined these data raised doubt in the whole concept of HDL as atheroprotective lipoprotein or, at least questioned, if HDL-C is the right biomarker to determine. Since HDL particles exert various atheroprotective properties independent of their cholesterol cargo, just measuring HDL-C might not accurately reflect the functional properties of HDL particles. Best known atheroprotective properties of HDL include cholesterol efflux capacity, anti-oxidative and anti-inflammatory functions [16-20]. Therefore, targeting HDL functionality might be a preferable strategy for therapeutic intervention to reduce the CVD risk [21]. However, before HDL function can be defined as novel therapeutic target, an improved understanding of the clinical value of different HDL functionalities is needed.

The importance of laboratory measurement of HDL functionality

HDL-C is a robust, consistent, and independent predictor for CVD [22] and, as such, has been included as a critical component in cardiovascular risk assessment tools both in Europe, e.g., in the PROCAM risk score, and in the US, reflected by the widely applied Framingham Risk Score [4,23]. Several pharmacological therapies including niacin or cholesteryl ester transfer protein (CETP) inhibitors which aimed to elevate the cholesterol level of HDL, did not result in significant reduction in CVD events [12-14,24-26]. This led to the conclusion that simply increasing HDL-C level may not be an effective strategy to reduce CVD risk [27]. HDL functionality might be considered as a potential biomarker for CVD and consequently a novel therapeutic target. [21,28-31] Patients with type 2 diabetes mellitus (T2DM) on maintenance hemodialysis are at a substantially increased risk of CVD [32-34]. Dyslipidemia characterized by moderately elevated LDL-C, high triglycerides and low HDL-C level is common in this population. However, in this patient group, the relationship between classical risk factors and CVD is inconsistent. Thus, HDL-C level is not a good predictive biomarker of CVD, but it is possible that alterations in the functionality of HDL might contribute [35-37]. Therefore, the study presented in chapter 2 examined if cholesterol efflux as a key metric of HDL functionality is predictive for

cardiovascular risk and overall mortality in patients with T2DM on hemodialysis participating in the 4D (Die Deutsche Diabetes Dialyse) Study. In this study (chapter 2), it has been shown that in a large and sufficiently powered cohort of patients with diabetes and End Stage Renal Disease (ESRD) on hemodialysis, HDL efflux capacity is not associated with cardiovascular events or mortality. These results extend previous observations that plasma HDL-C levels do not have a significant association with the risk of cardiovascular morbidity or mortality in ESRD [38,39]. However, our findings are in apparent contrast to the prevailing view in the cardiovascular field that, at least in cohorts with normal or only mildly impaired kidney function, the HDL cholesterol efflux capacity was associated with both subclinical atherosclerosis and obstructive coronary artery disease [28,29,40-42] For instance, in two larger prospective studies HDL cholesterol efflux capacity was inversely associated with cardiovascular events [28,29]. Notably, this effect of a key metric of HDL function was independent of HDL-C and was, in addition, suggested to be an even stronger predictor for CVD events than HDL-C level [29,41,43]. Although it should be noted that not all data on risk prediction by measuring cholesterol efflux are consistent with such a concept [44], specific characteristics of patients with ESRD may affect the outcome of the study. In the setting of renal failure, smaller cross-sectional studies indicated that anti-inflammatory [44,45], anti-oxidative [45], or endothelial health-promoting activities of HDL [46] are impaired to a similar degree. Furthermore, previous results using the same experimental set-up to determine cholesterol efflux indicated that cholesterol efflux capacity is not associated with cardiovascular or all-cause mortality in kidney transplant recipients that uniformly suffer from renal function impairment [47]. Possible reasons for the dysfunctional HDL in case of kidney disease could be due to modifications of the HDL particles such as oxidation, which negatively affects its anti-atherogenic properties and reverse cholesterol transport [48,49] and this could be accelerated by the presence of diabetes [44]. In fact, HDL of patients with renal disease carries a unique proteome and lipid composition linked to chronic systemic inflammation and potentially associated with a reduced anti-atherogenic ability of HDL [44]. In the same regard, it has been shown that HDL of patients with ESRD has lost its anti-inflammatory property due to direct consequence of enrichment with the acute–phase protein serum amyloid A (SAA) [50]. Subsequently, in this chapter (chapter 2), it was shown that SAA levels were significantly lower in patients with better HDL cholesterol efflux capacity. However, after correction for SAA, there was no significant correlation between cholesterol efflux capacity and SAA. Another result of this chapter was a potential effect modification of statin treatment efficacy depending on the HDL cholesterol efflux capacity despite the fact that the 4D study was not specifically designed to address such a question. It was shown that in patients within the first tertile of cholesterol efflux, atorvastatin reduced the risk for all cardiac events. It has been shown that atorvastatin had no statistically significant effect on the composite primary endpoint of cardiovascular death, nonfatal myocardial infarction, and stroke in the 4D study [51]. However, these data suggest that HDL cholesterol efflux capacity might be useful as a potential tool to identify subgroups of patients with ESRD that could benefit from statin treatment. Another possible explanation for the lack of a prospective association of cholesterol efflux with events in the 4D study could be that the pattern of CVD in ESRD patients differs from that in the general population with a lower percentage suffering “classical” myocardial infarctions due to atherosclerotic CVD [52,53]. Furthermore, the possibility cannot be excluded that CVD in the 4D patients had already advanced too far at the time point of inclusion making it less likely that a

better or worse HDL efflux capacity can still impact the further course of atherosclerosis. Clinical data on the impact of HDL efflux capacity or RCT during different stages of athetrosclerotic CVD are thus far lacking. Support for such a reasoning could come from previous work in renal transplant recipients (RTR), where the HDL efflux function had a significant effect on chronic graft loss [47]. Chronic graft loss is mainly due to transplant vasculopathy, which is largely caused by de novo atherosclerotic lesion formation in the kidney graft [54,55]. Here at the time point of transplantation arteries without atherosclerosis are subjected to a proatherogenic environment and HDL cholesterol efflux function can still have a beneficial effect. On the other hand, although less likely, it cannot be excluded that specifics of the experimental efflux system used in the present study precluded the identification of significant effects. This would require an evaluation of the predictive power of the current assay system in general population cohorts or other researchers using their J774 cells-based efflux system in ESRD or RTR cohorts. As mentioned previously the progressive reduction in kidney function is known to associate with a significant increase in CVD risk [56]. Cardiovascular morbidity and mortality is increased up to 30-fold in patients with ESRD [57]. Kidney transplantation is increasingly used to treat ESRD patients. However, RTR still suffer a 4-6-fold higher age-adjusted CVD mortality [58]. Also, dyslipidemia is highly prevalent in RTR and, similar to ESRD, does not explain the increase in CVD risk [54,59,60]. Similar to ESRD this clinical setting indicates that functional alterations of HDL could play a role. In addition, the pathophysiological mechanisms of CVD and chronic atherosclerosis-mediated graft failure are not fully clear [38,39,61,62]. In chapter 3 the predictive value of the HDL anti-oxidative capacity was examined for relevant long-term clinical outcomes in a prospective cohort of renal transplant recipients (RTRs). Our findings showed that in this prospective study, the anti-oxidative functionality of HDL is not a valid biomarker for CVD risk or all-cause mortality prediction. However, before extrapolating these results to the general population we need to consider that the nature of CVD in RTR may be different. Myocardial infarction due to occlusion of the coronary arteries is the most common type of CVD in the general population [63], whereas RTR experience a high incidence of sudden cardiac death and heart failure. Additionally, uremic cardiomyopathy due to a progressive decline in kidney function may contribute substantially to cardiovascular mortality in pre-transplant ESRD and subsequently in RTR [64]. However, still more prospective population-based cohort studies are warranted to clarify the value of HDL functionality in the prediction of cardiovascular events. In addition to cardiovascular and all-cause mortality, the study presented in chapter 3 examined whether the HDL anti-oxidative capacity at baseline (i.e. in patients with a functioning graft for more than one year) would be associated with future risk of chronic graft failure, since there is evidence that intragraft atherosclerosis plays an important role in the pathogenesis of chronic renal transplant dysfunction [65]. Our results showed that baseline anti-oxidative functionality predicts graft failure in RTR in crude and age-adjusted models. Unexpectedly though, a better anti-oxidative HDL function was associated with a higher risk of graft failure. A potential explanation could be that under conditions of a high oxidative stress and inflammatory load the HDL anti-oxidative properties increase and are thus just a mere reflection of an adverse environment rather than a pathophysiologically meaningful defense mechanism. Such an interpretation is supported by smokers having a significant increase in oxidative stress defense systems 66. Additional support could be derived from the observation that this correlation was lost once corrected for potential confounders,

most importantly eGFR and C-reactive protein (CRP). Both, a decline in kidney function and an increased inflammatory load are conditions of increased in vivo oxidative stress [67-70]. Taken together, the HDL anti-oxidative functionality does not have the potential to serve as an independent predictive biomarker for chronic graft failure. Therefore, more research, also including other functions of HDL, will be required to define the prospective value of HDL function for clinical outcomes in RTR. As mentioned previously individuals with T2DM have an increased prevalence of lipid abnormalities that contribute to higher rates of CVD, and typically have low HDL-C and an increased chronic inflammatory load [71]. Indeed, recent studies indicate that the age-adjusted relative risk of death as a result of CVD events is three-fold higher in T2DM patients than in the general population [72-77]. Activation of endothelial cells has been shown to initiate an inflammatory response characterized by the release of chemokines and adhesion molecules that direct monocytes to the affected region of the vessel wall [78,79]. An early step in this inflammatory process is the adhesion of monocytes to endothelial cells that have been injured or stimulated in the presence of oxidized or otherwise modified LDL to express several adhesion proteins, including vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and E-selectin [80,81]. The anti-inflammatory effects of HDL are thought to contribute to the protection from cardiovascular events [48]. One study showed e.g. that glycation of HDL adversely affects the anti-inflammatory properties of HDL in vivo in a preclinical model [82]. In chapter 4 we aimed to investigate whether T2DM impairs the HDL endothelium protective function (HDL anti-inflammatory capacity) and to delineate potential factors that impact on this metric of HDL functionality. Interestingly, the anti-inflammatory capacity of HDL was strongly impaired in the T2DM patients compared with controls. This occurred apparently early in the course of T2DM, since the patients had generally a good metabolic control. Consistent with the concept that glycation impacts HDL function, an impaired HDL anti-inflammatory capacity was related independently with plasma glucose levels [82]. Thus, it is possible that the impaired anti-inflammatory functionality of HDL contributes to the increase in the risk of atherosclerotic CVD associated with T2DM [48,83-85]. The finding of one previous study demonstrated that judged by a so-called HDL inflammatory index, HDL in patients with diabetes not only has lost its anti-inflammatory activity but it exerts even a pro-inflammatory activity [86]. SAA is a sensitive marker of inflammatory states and released in response to inflammation or infection [87-89]. Production of SAA is stimulated by pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) [89,90]. In plasma, circulating SAA is almost exclusively found within the HDL fraction [91,92]. With respect to SAA, one study showed that patients with T2DM have a chronic inflammatory condition that is characterized not only by increased levels of SAA but also with the loss of the anti-inflammatory and antioxidant function of their HDL. Moreover, this work found a significant correlation between HDL inflammatory index values and SAA concentrations [93]. Thus, the enrichment of HDL with SAA was suggested to explain decreased anti-inflammatory properties of HDL. However, in this chapter no significant association of SAA with the impaired HDL anti-inflammatory function was found. The absence of an independent association of the HDL anti-inflammatory function as observed in this study could have been due to the preferential inclusion of patients with good metabolic control of T2DM and relatively little systemic inflammation as compared to other cohorts. It has been suggested that CRP may contribute to the inflammatory process. Human recombinant CRP has been reported to increase induced VCAM-1, ICAM-1, and E-selectin in

some in vitro studies [70,94]. However, all of this work has been called into question by the preparation procedures of the recombinant CRP used in these respective studies; when using native human CRP isolated from plasma of donors, none of these effects could be replicated [95]. Alterations in HDL function could be related to various changes in HDL composition induced by inflammation [96]. The results in chapter 4 demonstrated that there was an association of impaired HDL anti-inflammatory capacity with the low-grade inflammatory marker hs-CRP (high-sensitivity C-reactive protein). The impaired anti-inflammatory activity of HDL might be the result of chronic hyperglycemic exposure. It was shown that this function of HDL was associated with fasting plasma glucose as well as glycated hemoglobin (HbA1c) [97,98]. Previously, our group showed in a limited number of patients that an impaired anti-inflammatory function of HDL predicts recurrent CVD events in myocardial infarction patients [99], but still large prospective studies would be required to explore the impact of this metric of HDL function on the future development of CVD in T2DM. In conclusion, the results of chapter 4 were consistent with the concept that T2DM exerts a substantial negative impact on the anti-inflammatory function of HDL, and this important metric of HDL function was already decreased even in mild hyperglycemia conditions. Only few studies so far have investigated more than one HDL function in a clinical setting at a time, however, it is still unknown whether measurement of one particular atheroprotective property of HDL is sufficient or more than one function of HDL should be evaluated. Therefore, in chapter 5 three main HDL functions (cholesterol efflux, anti-oxidative and anti-inflammatory properties) were assessed in patients with high estimated risk for coronary events participating in the PROCAM-CT study. However, no significant relation between HDL functionalities with PROCAM risk score, intima-media thickness (IMT), or Agatston score was found. It has been shown that coronary artery calcification (CAC) and IMT associated with future risk of cardiovascular diseases and they could be used as a predictor for future CVD risk [100,101]. Another study indicated that the CAC score is a strong predictor of CVD incidence, and might provide predictive information beyond the traditional risk factors in different ethnic groups [102]. In another study by Uthoff et al., sonographic measurement of the IMT in the common carotid artery was carried out and PROCAM risk scores were calculated; no correlation was found between PROCAM risk score and baseline IMT [103]. Thereby, it leaves the question if IMT is a good prediction factor in relation to the PROCAM risk score. Larger comparative studies would help to address this question. In this study (chapter 5) a positive relationship between HDL cholesterol efflux with total cholesterol, HDL-C, and apoA-I was observed, however, anti-oxidative and anti-inflammatory of HDL did not have any relationship with these measurements. Based on literature, the correlation of efflux with HDL-C and apoA-I is to be expected [29,40,47]. Furthermore, in this chapter, none of the three HDL functionalities determined was associated with PROCAM risk score in this high-risk study population. In principle, these results indicate that independent clinical information can be derived from measuring HDL function as compared to the PROCAM risk score. If determining HDL functionality discriminates actual risk for individual subjects better or worse than the PROCAM score can, however, not be decided based on the cross/sectional set/up of the current study. Future prospective studies would be required to specifically investigate this clinically relevant question. The basis for an observed lack of correlation between HDL function and PROCAM risk score might also be due to a relatively uniform impairment of HDL functions in this specific patient group, although a substantial spread in the experimental data was noted. Some

previous studies showed that significant differences in the functional properties of HDL may exist between patients and healthy control subjects [104,105]. For instance, it has been shown that HDL from acute coronary syndrome patients is defective and was not able to suppress TNF-α-induces VCAM-1 expression in endothelial cells [106]. Thus far, only with respect to cholesterol efflux capacity prospective data in the general population [28] and secondary prevention settings [107] have been generated. Of note, all available literature was generated with the use of one assay system. Still, not every study could confirm a prospective inverse association of efflux capacity with CVD risk, since even an inverse relationship was reported [108]. Overall, despite considerable evidence for functional impairments of HDL in clinically relevant settings of CVD, further large prospective studies are still warranted to shed light on the complex relationship among HDL functionality, HDL-C mass, and their clinical significance in risk-prediction or risk-modification in patients at high baseline risk for cardiovascular diseases. Such studies should ideally also include the determination of different HDL functionalities within one cohort as exemplified in the current chapter.

Potential clinical applications of HDL function assays

Measurement of HDL biological activities might help to define patients at increased risk of CVD. Cholesterol efflux capacity is viewed as a reflection of the efficiency with which HDL accepts cholesterol from peripheral tissues, most directly relevant for atherosclerosis from lipid-laden macrophage foam cells in the artery wall. This process represents the initial step of reverse cholesterol transport. In brief, cholesterol is removed in an interaction between HDL and the ABCA1, ABCG1 and SR-B1 transporters [109]. Therefore, a better understanding of this pathway may lead to new therapeutic targets in the prevention or cure of atherosclerosis. Chapter 2 and 5 of this thesis described research carried out on this specific metric of HDL in our aim to provide improved insights. Furthermore, anti-oxidative capacity of HDL can be impaired in several metabolic and inflammatory diseases [110]. HDL is associated with several anti-oxidant enzymes, such as paraoxonase 1 (PON1) and lipoprotein associated phospholipase A2 (Lp-PLA2) [111,112]. Thus, HDL plays a significant role in the prevention of lipid oxidation in LDL, a key consequence of oxidative stress and inflammation which leads to the formation and progression of atherosclerosis. Chapter 3 and 5 focused on measuring this function of HDL in patients with different diseases. Chronic kidney disease (CKD), including ESRD, patients are at high risk of CVD, which remains the major cause of morbidity and mortality in these patients' populations. Several factors are involved in the pathogenesis of atherosclerosis and CVD in these patients. These include oxidative stress, inflammation, endothelial dysfunction, vascular calcification and dyslipidemia [113]. Understanding the mechanisms responsible for HDL dysfunction are critical steps in inventing new effective therapies aimed at improving HDL function. Therefore, in Chapter 3 we aimed to evaluate the anti-oxidative function of HDL in renal transplant patients.

HDL has the ability to inhibit endothelial cell adhesion molecules such as VCAM-1 which facilitate the binding of mononuclear cells to the vessel wall and promote lesion development. HDL inhibits expression of cytokines such as TNF-α which mediate upregulation of endothelial adhesion molecules. The anti-inflammatory ability of HDL to inhibit adhesion molecule expression might be mediated by ApoAI and sphingosine-1- phosphate [28,29,47,107,108,114,115].

Therefore, there is a clear need for large prospective cohort studies to definitively prove a causal relationship between distinct aspects of HDL dysfunction and the risk of future cardiovascular events. To contribute to this important point, the association of HDL anti-oxidative capacity at baseline with future cardiovascular mortality, all-cause mortality, and graft failure was prospectively assessed in a cohort study of 495 RTRs (chapter 3). In addition, in chapter 2 the baseline HDL cholesterol efflux capacity was assessed in 1147 ESRD patients with diabetes (4D Study), a prospective study originally designed to explore the efficacy of atorvastatin treatment in patients with T2DM on hemodialysis.

Conclusion and future directions

Despite a number of studies addressing functional aspects of HDL, the importance of HDL functionality for cardiovascular risk is still largely unknown. The research described in this dissertation helps to better understand how HDL functionalities can be measured in order to gain further insight into the relationship between HDL functions and the risk of CVD. However, there are many areas that warrant further research.

Cholesterol efflux capacity might be useful as a potential risk assessment to identify subgroups of patients with ESRD that could benefit from statin treatment (**chapter 2**). Otherwise, cholesterol efflux does not appear useful to predict CVD mortality in settings of impaired kidney function. Also, the anti-oxidative capacity of HDL was not helpful in predicting future cardiovascular or all-cause mortality in patients with decreased kidney function, namely RTRs. It also proved to have only a very limited value to predict graft failure in this group of patients (**chapter 3**). Still studies exploring if the anti-oxidative capacity of HDL is a predictor of CVD in the general population need to be conducted. T2DM relatively early in the course of the disease exerts a substantial negative impact on a critical atheroprotective function of HDL. Here, prospective studies are urgently needed, since the anti-inflammatory function appears to be a very sensitive metric. In addition, the independent association of the anti-inflammatory capacity of HDL with hyperglycemia stresses the importance of tight metabolic control in T2DM (**chapter 4**). Overall, these findings indicate that important information can be derived from measurements of HDL functionality with respect to different forms of CVD. However, there is still a lack of uniformity of procedures for the determination of HDL functionality across laboratories. As HDL functionality is an emerging topic of interest for both clinicians and researchers, there is an increased need to improve standardization of assays for HDL functionality. For instance, standardized HDL isolation and characterization methods need to be developed. Also, different assay conditions for the same HDL function need to be compared with respect to their clinical relevance. Ultimately, the question whether HDL functionality can predict future cardiovascular events in the general population remains unanswered. Therefore, future large prospective cohort studies are required to conclusively test a potential causal relationship between HDL functionality and disease.

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