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General discussion and future perspectives
INTRODUCTION

Cardiovascular disease (CVD) is the leading cause of mortality globally [1]. Within Europe, 45% of all mortality is caused by CVD [2]. Mortalities related to CVD include myocardial infarction and ischemic stroke. The major risk factor for CVD is dyslipidemia. However, behavioral factors and co-morbidities related to CVD such as the metabolic syndrome are also contributing to the risk of CVD events [3,4]. Although reducing behavioral risk factors such as reducing smoking and increasing physical exercise in patients is challenging, the progress in government funded life-style interventions is projected to induce a halt to the ever increasing incidence of CVD in patients [3]. However the increasing incidence of type 2 diabetes (T2D) and increasing body mass index (BMI), counteract these trends [1]. Current pharmacological intervention focusses on lipid lowering via statin treatment. Unfortunately, statins reduce the CVD-associated mortality burden with only 25% [5]. Identifying novel targets for therapeutic application is thus essential to reduce the residual risk present in current and future patients. We pre-selected potential targets based on a combination of genetic, transcriptomic and proteomic approaches in cardiovascular and metabolic disease. In this thesis, we validated these putative targets in a preclinical setting.

PUTATIVE TARGETS IN CARDIOVASCULAR AND METABOLIC DISEASE

Stabilin-1 as a putative therapeutic target

To generate specific and efficient novel therapeutic strategies, the understanding of the major underlying pathology of CVD, atherosclerosis, is of key importance. Atherosclerosis is a pathological condition of the arteries which develops over time. A complex interplay between dyslipidemia and chronic inflammation results in the development of atherosclerotic lesions in medium and large-sized arteries [6]. One of the hallmarks of atherosclerosis is the accumulation of modified lipids in the arterial wall. Macrophages in the vessel wall become foam cells by the excessive uptake of modified lipids from the environment [7]. Scavenger receptors present on macrophages facilitate the uptake of lipids and subsequently mediate foam cell formation [8]. We have selected the scavenger receptor Stabilin-1 (Stab-1) as a putative therapeutic target for validation based on differential expression during development of murine atherosclerotic lesions and ex vivo foam cells. Furthermore, genetic variations were identified in the Stab-1 gene in a family with premature atherosclerosis (J.C. van Capelleveen, G.M. Dallinga-Thie and K.G. Hovingh, AMC Amsterdam, unpublished data). In Chapter 2, we investigated the effect of Stab-1 in macrophages on atherosclerotic lesion formation in mice. Interestingly, the absence of Stab-1 did not affect in vivo foam cell formation. In line, atherosclerosis susceptibility was not affected. Thus, despite that the genetic and transcriptomic data suggested a possible role for Stab-1 in the pathogenesis of atherosclerosis, we could not
validate this in our murine model for the disease. This discrepancy could be a consequence of the approach to identifying the target or it could be intrinsic to the in vivo functioning of the target. When dealing with microarray data it is important to be aware of the high false positive rates which arise from the multiple statistical testing. The field of bioinformatics is trying to keep up with the developments in genetic tools in order to correctly analyze and interpret the large datasets [9]. The development of novel computational models for analyzing microarray data will help reduce the false positive rates in the future [9,10]. From a biological point of view, it is important to note that functional redundancy has been shown for other macrophage scavenger receptors, i.e. SR-A1 and CD36. As such, the effects of deficiency of one or more macrophage scavenger receptors on atherosclerosis development in murine models remains controversial [11]. Moreover, this functional redundancy forms a challenge to employing the downregulation of scavenger receptors as a therapeutic approach.

**Proteoglycan 4 as a putative therapeutic target**

An example showing that genetic and proteomic association studies are able to predict causality is proteoglycan 4 (Prg4). We found a marked induction of Prg4 expression in initial atherosclerotic lesion formation in mice, based on which we selected this proteoglycan as a putative therapeutic target for validation in our murine models for atherosclerosis. Interestingly, in a family with premature atherosclerosis, variations were found in the Prg4 gene associating with the disease (J.C. van Capelleveen, G.M. Dallinga-Thie and K.G. Hovingh, AMC Amsterdam, unpublished data). Proteoglycans are macromolecules involved in the retention of modified lipoproteins in the arterial wall and the interaction of lipoproteins with cells [12,13]. In Chapter 3, the role of Prg4 in foam cell formation, cholesterol efflux and atherosclerosis was investigated in mice. We found that Prg4 deficiency in macrophages leads to increased susceptibility to foam cell formation as compared to wild-type (WT) cells. The foam cell reducing effect of macrophage Prg4 seems to depend on a positive impact of the protein on cellular cholesterol efflux. The detrimental effects of macrophage Prg4 deficiency on macrophage cholesterol efflux were observed not only in the presence of HDL and ApoA1, likely as a consequence of reduced SR-BI and ABCA1 expression, but also in the absence of added extracellular acceptors. An endogenously produced cholesterol acceptor is ApoE [14]. Notably, Lucas and colleagues showed that a substantial fraction of ApoE produced by macrophages is sequestered in an extra cellular network of proteoglycans [15]. Furthermore, this extracellular ApoE pool facilitates the efflux of sterols from macrophages in the presence and absence of an added acceptor [16]. It could be proposed that Prg4 is involved in these processes and thereby influences macrophage cholesterol efflux. However, it should be noted that the effect sizes of macrophage-specific Prg4 deficiency on foam cell formation and cholesterol efflux were small and atherosclerotic lesion development was not affected in this model. We observed additional minor effects of Prg4 on macrophage inflammatory response. Based on these pleiotropic effects of Prg4 and the fact that Prg4 is an ubiquitously
expressed and secreted proteoglycan, we anticipated that the quantitative contribution of bone marrow-derived Prg4 to the total serum Prg4 levels is minimal. This led us to the hypothesis that the overall availability of Prg4 in the serum rather than the source of Prg4 production is the determining factor in atherosclerotic lesion formation. In line, total-body Prg4 deficiency does affect atherosclerotic lesion susceptibility in mice (as described in Chapter 4), although the underlying mechanisms remain to be elucidated. These combined observations highlight the complexity of studying atherosclerosis, as the development of atherosclerotic lesions is the result of both local disease mechanisms and systemic disturbances.

The processes involved in CVD and metabolic syndrome are intimately linked. This is highlighted by the fact that Prg4 is also positively associated with increased weight, increased dyslipidemia and increased HOMA-IR in obese human subjects [17–19]. In Chapter 5 we showed that under obesogenic and diabetic circumstances, Prg4 KO mice develop lower plasma lipids, significantly better glucose handling tolerance as well as a strong trend towards an improved HOMA-IR score. This mirrored the associations found in humans, highlighting a potential translational value of this target. One of the characteristic diseases associated with metabolic syndrome is the accumulation of triglycerides in the liver, termed hepatic steatosis [20]. There is a strong association found between insulin resistance and hepatic steatosis suggesting that hepatic steatosis is a consequence of disturbed insulin regulation [21,22]. Moreover, insulin resistance is suggested to be a causal factor in white adipose tissue inflammation [23]. In our experiments we showed that Prg4 deficient mice had improved glucose handling, decreased hepatic steatosis and decreased adipose tissue inflammation. These independent effects could be mediated via separate processes. However, it can also be proposed that the improved insulin handling is a common causal pathway. The underlying mechanisms showing a causal role for Prg4 in insulin resistance are yet unknown.

In summary, throughout our experiments we found pleiotropic effects of Prg4 deficiency in multiple tissues. Our data in total body Prg4 deficient mice highlight the complexity of studying the impact of an ubiquitously expressed, secreted factor with pleiotropic effects in the context of cardiovascular and metabolic disease. Furthermore, until the underlying function(s) of Prg4 are elucidated, Prg4 remains a precarious therapeutic target. In addition, although Prg4 deficiency alleviates metabolic syndrome, it aggravates atherosclerosis. It would therefore be valuable to further validate the cardiovascular disease susceptibility and underlying mechanisms in a combined atherosclerosis / metabolic syndrome mouse model.

**Protein arginine methyl transferase 3 as a putative therapeutic target**

Protein arginine methyl transferase 3 (PRMT3) was selected as a potential therapeutic target based on differential expression upon macrophage foam cell induction. Moreover, its presence and transcriptional regulation was validated in developing atherosclerotic lesions in mice using a microarray. Upon literature review, we also found that PRMT3 interacts with Liver X Receptor (LXR), modulating its activity as a cofactor for LXR-mediated transcrip-
tion [24]. Since LXR is a well-defined target in both cardiovascular and metabolic disease [25], we investigated the effects of PRMT3 inhibition in the context of cardiovascular and metabolic disease in Chapter 7. LXR is a transcription factor that stimulates macrophage cholesterol efflux via upregulation of the ATP-binding cassette transporters A1 and G1. Therefore, LXR agonists are widely investigated as potential therapeutic agents for the treatment of cholesterol-driven diseases such as atherosclerosis [26,27]. However, LXR also regulates de novo hepatic lipogenesis via activation of the transcription factor sterol regulatory element-binding protein 1c (SREBP-1c) [28–30]. As a result, LXR nuclear receptor activation by the use of LXR agonists leads to unwanted hepatic steatosis [31,32]. This steatotic effect of LXR agonism has hampered the development of LXR agonists for clinical use in humans. In our experiments, the hyperlipidemic ApoE KO mouse model which is commonly used as a model for studying atherosclerosis, developed hepatic steatosis upon challenge with a Western-type diet. PRMT3 inhibition abrogated this hepatic steatosis, without affecting atherosclerosis susceptibility. This research suggests that inhibition of PRMT3 can uncouple the LXR-driven lipogenic effect and cholesterol modulating effect. In Chapter 6 we furthermore showed that the reduction of the hepatic steatosis is a direct effect of the PRMT3 inhibitor on LXR transcriptional activity in the liver. More specifically, we showed that C57Bl/6 mice treated with the PRMT3 inhibitor were protected from the development of hepatic steatosis by blunting the upregulation of the LXR-target genes involved in hepatic lipogenesis. This is in line with other studies implicating a role for LXR in hepatic steatosis [33]. However, more research is needed to completely uncover the underlying mechanisms of hepatic steatosis and the role of PRMT3 in this process. Since hepatic steatosis is a risk factor for cardiovascular disease independent of the other classical risk factors associated with the metabolic syndrome [34], PRMT3 inhibition and LXR function are valuable topics for future research. Moreover, LXR is also highly expressed in white adipose tissue and a local role for LXR in adipocyte lipogenesis has been suggested [35]. Stimulating adipocytes with a LXR agonist, increases adipocyte lipid accumulation [36]. In our experiments described in Chapter 7, inhibition of PRMT3 resulted in resistance to body weight gain. The adipocytes in these mice tended to be smaller with a decreased lipid content. This suggests that LXR in white adipose tissue metabolism could also be modulated using the PRMT3 inhibitor. So, from the data described in Chapter 6 and 7 we can conclude that PRMT3 inhibition in combination with LXR stimulation could be a valuable therapeutic approach to overcome the development of hepatic steatosis in the context of effective LXR-mediated lowering of atherosclerosis burden. Moreover, the PRMT3 inhibition-induced resistance to body weight gain could be a supplementary beneficial effect of this novel therapeutic strategy.
FUTURE PERSPECTIVES

In this thesis we validated putative targets for cardiovascular and metabolic disease in murine models (summarized in figure 1). Selection of the targets was based on genetics, transcriptomics and/or proteomics combined with literature research in both humans and mice. Upon validation, only one of the targets affected the primary outcome according to the original hypothesis. This clearly indicates that there is room for improvement in the selection of putative therapeutic targets for validation. Originally, the majority of target finding strategies using genetic approaches relied on identifying families with pronounced disease burden [37]. Genetic analysis of families has led to the discovery of many genes for Mendelian diseases and traits [38]. Although identifying genes in more complex diseases such as cardiovascular disease provides challenges using this approach, solid progress has been made, greatly advancing the field of cardiovascular disease. The latest successes were made when gain of function mutations in the proprotein convertase subtilisin/kexin type 9 (PCSK9) were shown to be associated with augmented LDL cholesterol levels and increased cardiovascular risk in a French family with autosomal dominant hypercholesterolemia [39]. This initial finding has

![Figure 1: Summary of the targets covered in this thesis and their effects in cardiovascular and metabolic disease.](image)

Although the different pathological processes are interlinked, it is a combination of these interactions and local processes determining the disease outcome. WAT: White adipose tissue, n.d.: not determined. Adapted from Magge et al., Pediatrics 2017
been rapidly translated into a functional therapeutic strategy, where PCSK9 inhibition is applied to effectively lowering LDL levels in patients [40].

Advances in genotyping technology has made it possible to screen large genetic datasets for associations of single nucleotide polymorphisms (SNPs) with disease outcome in so-called genome wide association studies (GWAS). This results in a vastly growing output of SNPs showing associations with cardiovascular outcome [41]. To date, close to 10,000 strong associations between genetic variants and complex disease traits have been identified [42]. Despite their potential, the variants identified thus far only account for less than 10% of the heritable risk. In order to effectively select putative targets, novel integrated approaches such as systems biology and systems medicine are imperative. These kinds of network analyses use pathway information and data from multiple measurement platforms, tissues and species [43]. This allows scientists to more closely model the complex, multi-layered disease taking environmental factors as well as multiple physiological systems into account. Moreover, to gain more insight in the disease processes and especially in the more complex traits, researchers are now also focusing on coincident associations [44]. These are the overlapping targets from GWAS studies in multifactorial diseases. This latter approach reduces the amounts of hits to be validated in disease models for complex diseases such as cardiovascular diseases and increases the potential relevance of the investigated genes. In another, more pharmaceutical-driven approach, the most promising candidate targets from genetic association studies can be selected by cross-referencing the GWAS candidate genes with a list of genes encoding drugs and druggable targets (the so called “druggable genome”) [45].

Despite this rise in in silico and computational modeling in target identification and selection, preclinical validation of putative targets in vivo remains important. Target validation can be divided in multiple levels of credibility, depending on the complexity and the disease relevance of the model used for validation [46]. Therefore, testing novel targets in cardiovascular and metabolic disease is preferentially executed in models representing a metabolic syndrome phenotype in combination with cardiovascular disease. The targets described in this thesis were all validated in murine models for disease. Although we found promising effects of these targets on disease outcome in our research, it is important to note that only one third of all animal based research is translated to human randomized trials [47]. Therefore care must be taken when directly translating these results to a human setting. In recent years, progress has been made to humanize models for validation of potential therapeutic targets. In an organ-on-a-chip model, cellular interactions as well as the physiological environment of specific tissues such as the white adipose tissue and the arterial wall can be mimicked [48,49]. Despite the major advancements in these in vitro and ex vivo models, the pathological processes in the cardiovascular disease and metabolic disorders are not fully understood, which limits the accuracy of models outside of a complex organism. So, until alternatives are developed combining the complexity of a whole organism with human physiological characteristics, animal models remain the best option for target validation.
In this thesis, we discussed the importance of the interactions of cardiovascular disease and metabolic disease. Many of the characteristics of these multifactorial diseases overlap. Our data on Prg4 as a potential target confirms this, showing effects of Prg4 both on atherosclerosis and metabolic syndrome. Large groups of patients using statins are still at risk for cardiovascular events [50,51]. This ‘residual risk’ should be addressed by additional treatment. Ideally, lifestyle intervention therapy would reduce the risk factors associated with both cardiovascular disease and metabolic syndrome. However, the effects of intensive lifestyle intervention on cardiovascular disease outcome are disappointing [52]. Alternatively, it would be interesting to use combination treatment of lipid lowering drugs such as statins with drugs increasing metabolic health pharmacologically. Combination therapy of the statin atorvastatin and the anti-diabetic drug metformin showed an additional reduction of atherosclerosis burden in a rabbit model for cardiovascular disease [53]. In addition, the perspective of target finding in cardiovascular disease could be broadened to include targets affecting both cardiovascular as well as metabolic syndrome. An example of such a target is the adipose tissue-derived hormone adiponectin [54]. Due to its anti-inflammatory, antioxidant, anti-atherogenic, pro-angiogenic, vasoprotective and insulin-sensitizing properties, adiponectin is proposed as a therapeutic target in cardiovascular and metabolic disease [55]. Interestingly, obesity and related pathologies decrease the endogenous adiponectin production [56], providing a practical therapeutic approach by increasing the levels of this adipokine. However, further validation of this target and its use in cardiovascular and metabolic disease is needed.

In conclusion, with the studies presented in this thesis we investigated the potential of 3 putative novel therapeutic targets, including Stab-1, Prg4, and Prmt3 in cardiovascular and metabolic disease. The results (1) highlight the complexity of both multifactorial diseases and potential overlapping mechanisms and (2) suggest that a more integrative approach in cardiovascular and metabolic disease research could be beneficial for therapeutic target identification and validation.
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