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Chapter

11

Summary, Discussion and
Future Perspectives

Part 1

Part 1 of this thesis focused on the assessment of the safety and efficacy of apoB synthesis inhibition with mipomersen, a novel strategy to lowering low-density lipoprotein cholesterol (LDL-c).

In **Chapter 2** we start with a summary of the results of the most relevant preclinical and clinical studies investigating mipomersen, an apoB synthesis inhibitor for the lowering of LDL-c, available thus far. In all these studies mipomersen administration was shown to effectively reduce LDL-c and was overall well tolerated with injection site reactions, flu-like symptoms and increases in liver transaminases as the main adverse events. Clinically relevant increases in intrahepatic triglyceride (IHTG)-content were not reported.

In Chapter 3, 4 and 5 we present results of randomized double-blind placebo controlled Phase II studies in which we evaluated the safety and efficacy of mipomersen. Whereas statins are first line medication for the prevention of cardiovascular disease (CVD), future use of mipomersen will most probably be restricted to patients with very high levels of LDL-c despite optimal statin therapy. In **Chapter 3** we therefore studied the efficacy and safety of mipomersen when added to conventional lipid-lowering therapy in 44 patients with heterozygous familial hypercholesterolemia (FH). Patients received 8 doses subcutaneously over a 6-week treatment period. Mipomersen produced significant reductions in LDL-c (-21%) and all other atherogenic apoB-containing lipoproteins and there were no clinically significant drug-drug interactions. The positive results of this study showing significant efficacy of mipomersen on top of potent statin therapy, paved the way for further development of mipomersen as part of the LDL-c lowering treatment strategy in patients with severe LDL-c elevations, including patients with FH.

Since profound increases in IHTG-content were observed following another compound targeting hepatic secretion of VLDL (MTP-inhibitors) we questioned whether mipomersen treatment would also result in hepatic steatosis. **Chapter 4** describes the results of a study specifically designed to investigate the impact of mipomersen on IHTG-content in patients with FH. We found that mipomersen administration for 13 weeks to subjects with FH does not result in a significant increase in IHTG-content. However a trend towards an increased IHTG-content was observed accompanied by one subject progressing into new-onset hepatic steatosis in spite of a relatively modest group size, exclusion of subjects at increased risk of developing hepatic steatosis and less profound lipid reductions than those reported in previous clinical trials with mipomersen. It was concluded that the effect of mipomersen on IHTG-content needed to be further evaluated,

following prolonged treatment duration and in a larger number of subjects including those at increased risk of hepatic steatosis.

In **Chapter 5** we investigated the safety and efficacy of mipomersen in another patient group that may benefit from alternative LDL-c lowering: statin intolerant patients at high risk for CVD. Twenty six weeks of treatment with 200 mg of mipomersen resulted in significant reductions in LDL-c with a mean of 47%. Only 4 of the 33 subjects discontinued treatment prematurely due to study related adverse events. Persistent liver transaminase increases ≥ 3 x upper limit of normal (ULN) in subjects assigned to mipomersen treatment (33%) were twice as common compared with previous clinical trials. When IHTG content was measured in 1 placebo treated subject and in 14 mipomersen treated subjects with increases in alanine aminotransferase (ALT) of >2 x ULN, hepatic steatosis was detected in 1 subjects from the placebo treated group and in 12 subjects from the active treatment group with a significant reduction in IHTG-content following discontinuation of treatment (-17.7%). Liver needle biopsy performed in 2 subjects, detected severe hepatic steatosis with minimal inflammatory changes. Whereas mipomersen administration resulted in profound hepatic steatosis, we suggested that more progressive liver disease may be absent. We concluded that pending long term safety effects, apoB synthesis inhibition may offer an alternative therapeutic strategy for patients at high risk for CVD for whom currently no other therapeutic options are available.

The antisense technology in general has the ability to inhibit unique targets with high specificity. Furthermore antisense drugs have an optimal distribution within liver, kidney and atherosclerotic plaques, and lack interaction with the cytochrome P450 system. Therefore, the antisense technology may be an exciting and valuable novel therapeutic modality. Mipomersen, the antisense drug described in this thesis, is the most advanced of antisense inhibitors for the treatment of CVD. Mipomersen has shown impressive efficacy results, comprising profound reductions in LDL-c and all other atherogenic lipid particles, including Lp(a). But, although short term and longer term efficacy data are encouraging, the safety of this compound remains to be further investigated. Future studies should be performed to minimize the incidence of injection site reactions (ISR), since ISR may impact long term adherence. The nature of hepatic enzyme increases should be sort ought since it is still unclear whether ALT increases are a direct pharmacological effect or if they are related to hepatic fat accumulation. And, most importantly, the effects of mipomersen administration on IHTG-content should be thoroughly evaluated. In this thesis we describe profound hepatic steatosis following mipomersen treatment in statin intolerant patients. Whereas hepatic steatosis was not detected in previous clinical trials it will be of interest to evaluate what may explain for this discrepancy.

Has hepatic steatosis previously been underreported? Are statin intolerant patients at increased risk for hepatic steatosis compared to hypercholesterolemic patients or patients with FH? Do statins reduce efficacy of mipomersen and are statins protective for fat accumulation? Phase III studies with mipomersen in patients with heterozygous FH and severe hypercholesterolaemia are incorporating MRS to measure IHTG-content. These studies, in addition to two open-label extension trials, which are monitored regularly by a Data Safety Monitoring Board, may provide answers to some of these questions.

In chapter 5 we propose the concept that hepatic steatosis without metabolic sequelae may distinctly differ from NAFLD and may not progress to more severe liver disease. This would imply that hepatic steatosis following apoB synthesis inhibition may not result in fibrosis or cirrhosis provided metabolic disturbances are absent. But evidence for this concept is thus far minimal and it needs further validation in extended safety studies including biopsy data.

In conclusion, long term tolerability and safety data will be crucial for the further development of mipomersen. Awaiting long term safety effects, administration of mipomersen should continue but only under carefully monitored conditions and only to patients at high risk for CVD in whom no alternative therapeutic strategies are available and in whom therefore the benefit may outweigh the risk.

Part II

In part II of this thesis we described the results from research on 2 rare dyslipidemic disorders and the metabolic syndrome. **Chapter 6** describes the results of a study investigating the relationship between hepatic steatosis and insulin resistance. Hyperinsulinaemic euglycaemic clamp studies have shown that increased IHTG-content strongly correlates with insulin resistance across a large range of percent liver fat. In contrast, we show with a 2-step hyperinsulinemic euglycemic clamp using stable isotopes that in patients with familial hypobetalipoproteinemia (FHBL), who have severe hepatic steatosis, both hepatic and peripheral insulin sensitivity does not differ from healthy matched controls. We conclude that hepatic steatosis per se is not directly related to insulin resistance and suggest that liver fat accumulation without metabolic sequelae distinctly differs from NAFLD. Our observations are important for further understanding of the mechanisms underlying the metabolic syndrome. However many questions remain unanswered. For example, what other factors beyond TG accumulation are responsible for the close relation between hepatic steatosis and insulin resistance as observed in epidemiological studies? Comparing microarray expression profiles in liver biopsy material from patients with different types and degrees of hepatic steatosis (e.g.

NAFLD, NASH, hepatitis, mipomersen), may be a valuable method to investigate this research question. In addition, this strategy may also identify risk factors for more progressive liver disease. These findings would especially be of interest for the further development of the apoB synthesis inhibitor, mipomersen, described in Part 1.

In **Chapter 7** we show an increase in the incidence of familial partial lipodystrophy (FPLD) in non-obese patients (BMI ≤ 27 kg/m²) with type 2 diabetes mellitus and marked insulin resistance (>100 U insulin/day). Amongst 5221 patients with type 2 diabetes mellitus we identified 5 patients with lipodystrophic features. One of these patients harboured a novel heterozygous mutation (Y151C) in *PPARG* which was functionally assessed. We show that haploinsufficient mutations may be sufficient to produce a lipodystrophic phenotype and suggest that thorough evaluation for FPLD should be considered in non-obese diabetic patients with marked insulin resistance. Of interest, analysis of the pedigree revealed that clinical features of the affected subjects varied considerably. In contrast to the index patient, the proband's sister and her son were only mildly affected, with clinical features such as hypertriglyceridemia (HTG) and hypertension but no diabetes mellitus. This clinical heterogeneity within one kindred is consistent with observations from other families with *PPARG* mutations and may be the result of both genetic as well as environmental factors. It will be of interest to follow the clinical course of the affected family members in our kindred to determine if they will eventually develop a phenotype similar to the index patient.

Identification of FPLD patients enables the discovery of novel causative genes that eventually may lead to new treatment targets. In addition, studying these rare patients at the extreme end of the phenotypic spectrum may provide insights into the role of adipose tissue in metabolic homeostasis in general and may help to improve our understanding of the more common metabolic syndrome.

Part III

Part III of this thesis describes research on triglycerides (TGs). In **Chapter 8** we evaluated the risk for coronary heart disease (CHD) in patients with metabolic dyslipidemia (elevated plasma TG and low HDL-c levels). Men with metabolic dyslipidemia had an increased risk for CHD compared to men with normal HDL-c and normal TGs (HR: 1.63; 95% CI: 1.42-1.87) and risk remained significant after adjustment for LDL-c and other metabolic syndrome risk factors. Of interest, we found that risk of developing CHD was similar in men with low HDL-c and high or normal TG levels. We also observed that in men with normal HDL-c, TG levels

were not predictive of CHD. These observations suggest low HDL-c levels to be an important CHD risk factor in men.

Among women, metabolic dyslipidemia was also associated with increased CHD risk (HR: 1.80; 95% CI: 1.49-2.18). Although risk in women was higher compared to men, the association was lost when the model was additionally adjusted for other metabolic syndrome risk factors. In men and women Kaplan-Meier survival curves according to HDL and TG levels revealed that participants with metabolic dyslipidemia had poorer survival ($p < 0.001$).

We conclude that in both men and women metabolic dyslipidemia is a predictor of CHD independent from LDL-c. Furthermore the relationship between metabolic dyslipidemia and CHD risk is independent from the other risk factors associated with the metabolic syndrome in men, but not in women. These findings confirm that patients with low HDL-c and high TG levels should be protected against CHD. In addition these results support the concept that risk factors of CHD are interdependent in the metabolic syndrome and that the metabolic syndrome might present cardiovascular risk beyond what can be explained by its individual components.

Chapter 9 describes the results of sequencing the promoter and coding regions of TG candidate genes in 86 consecutive patients referred to our lipid clinic for evaluation and treatment of severe hypertriglyceridemia (HTG). In 46 (54%) patients we identified rare DNA sequence variants including 19 variants in *LPL*, 1 in *APOC2*, 2 in *APOA5*, 4 in *GPIHBP1* and 8 in *LMF1*. In 22 patients (25%) we identified only common variants in *LPL* and *APOA5* whereas in 18 patients (21%) not a single mutation was found, suggesting the involvement of unknown genes. *In vitro* validation revealed that the mutations in *LMF1* were not associated with compromised LPL function. Consistent with this, five of the eight genomic variants in *LMF1* were also found in a control population and therefore cannot account for the severe HTG phenotype. We conclude that genetic variation in *LPL* is the most important contributor to the phenotype of severe HTG. In contrast, mutations in *GPIHBP1*, *APOC2* and *APOA5* are rare whereas mutations in *LMF1* do not seem to contribute substantially. These results show that in carefully selected groups with severe HTG, molecular diagnosing is feasible and effective. This finding may be of clinical relevance since molecular diagnosing may in the near future allow for individually tailored therapeutic strategies in severe HTG patients. In future studies it will be of interest to perform whole exome sequencing in those patients without functional mutations to elucidate the causal mutation.

In **Chapter 10** we performed a non-biased GWAS in 463 patients with HTG and 1197 controls. GWAS revealed that common variants in *APOA5*, *GCKR*, *LPL* and *APOB* were associated with the HTG phenotype. Resequencing these genes identified a significant difference in the presence of variants between patients

and controls: in 438 HTG patients we identified 154 rare missense or nonsense variants whereas in 327 controls 53 variants were identified ($p=6.2\times 10^{-8}$). These findings corresponded to a carrier frequency of 28.1% of HTG patients and 15.3% of controls ($p=2.6\times 10^{-5}$). A more restricted analysis, including only rare variants found exclusively in either HTG patients or controls and removing all previously reported non functional variants, revealed 47 variants in HTG patients compared to 9 variants in controls ($p=2.4\times 10^{-5}$); this corresponds to a significantly increased carrier frequency of 10.3% in HTG patients compared to 2.8% in controls ($p=4.4\times 10^{-5}$). A logistic regression model including clinical variables and both common and rare genetic variants explained 41.6% of total variation in HTG diagnosis: clinical variables explained 19.7%, common genetic variants in 7 HTG-associated loci explained 20.8% and rare genetic variants in 4 HTG-associated loci explained 1.1%. Thus we demonstrate that loci found to be associated with HTG by GWAS using common variants also harbour a significant excess of rare variants. All together these findings confirm the hypothesis that both rare and common variants in TG-associated genes together contribute to the phenotypic heterogeneity underlying HTG.

Future Perspectives

LDL-c lowering

LDL-c is the mainstay of CVD prevention. But, despite the presence of effective LDL-c lowering drugs, the number of patients unable to reach LDL-c targets as defined by the current guidelines, is expanding. Therefore, alternative LDL-lowering modalities are urgently required. In the present thesis, we presented results from clinical trials evaluating the safety and efficacy of mipomersen, an apoB synthesis inhibitor. Whereas we show mipomersen to effectively lower LDL-c, its safety remains to be further elucidated in prolonged safety studies prior to broader use of this compound. In fact positive results in future safety studies will be crucial for further development of mipomersen. But apoB is not the only promising novel target for drug therapy and antisense is not the only novel therapeutic modality. For example MTP inhibitors, PCSK9 inhibitors (antibodies, siRNAs and lock-nucleic acid ASOs) as well as thymimetics such as eprotrirome have demonstrated significant reductions in LDL-c levels in clinical or preclinical trials¹⁻³. Further studies examining the efficacy and safety of all of these agents are needed to determine their potential clinical applicability.

Metabolic syndrome

Whereas LDL-c will remain the mainstay of CVD prevention, the drastic increase in the incidence of obesity will expand the role of metabolic syndrome risk factors. The battle against overeating and physical inactivity will however not be easy to win since changing life style and behavior has been shown to be extremely difficult. Currently there are several effective therapeutic options available to target some of the individual components of the metabolic syndrome however pharmacological agents for the prevention or management of the metabolic syndrome itself have been limited and unsatisfactory. Most of these drugs address weight reduction by targeting appetite at the central nervous system. But higher incidence of depressive symptoms or cardiovascular side-effects (rimonabant, orlistat, sibutramine) has limited the use of these compounds⁴. Thus, there is clearly a significant unmet medical need for safe and effective therapies to prevent the metabolic derangements that are associated with increased central adiposity. Of interest, currently thymomimetic analogues are in development to provide such an overall treatment strategy⁵.

In conclusion insight into the metabolic consequences of obesity is particularly relevant for the development of novel treatment strategies directed towards the prevention and treatment of metabolic syndrome sequelae. Studying patients with rare monogenetic phenotypes, such as patients with FPLD or FHBL may improve the understanding of this complex disorder.

Triglycerides

Plasma TG levels are re-emerging as an independent risk factor for CVD. But treatment, especially of patients with moderate to severe HTG, is often challenging. More complete understanding of pathways involved in TG metabolism may therefore help to identify new directions for therapeutic interventions. In addition, identified genetic variants may improve risk prediction. For example genetic risk scores may predict the risk of developing HTG or CVD when added to existing risk scores, which could both prompt early intervention.

Whereas recent GWAS studies have identified known and novel loci associated with HTG, genetic variation at these loci explains only 10% of overall TG variation within the population. Novel identified loci may have even smaller effect sizes. Therefore, since GWAS may be reaching its limit for detecting novel TG associated loci, future studies should focus on alternative genetic strategies, such as family studies, animal studies, or rare variant sequencing, to provide more complementary information for understanding pathways in TG metabolism.

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