Apolipoproteins A-I and A-V as gene therapeutic targets to intervene in lipid metabolism
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General discussion and implications
This thesis focuses on the potential of recombinant adeno-associated virus (AAV) to express therapeutic proteins in the liver to modify lipid and lipoprotein metabolism. In Chapters 2 and 3 we describe the potential of gene therapeutic approaches in lipid disorders and the optimization of AAV-mediated hepatic expression of therapeutic proteins, respectively. This latter optimized gene delivery protocol was subsequently used to explore the therapeutic potential of apolipoprotein (apo) A-I overexpression (chapters 4 and 5). Finally, in chapters 6-8, we examined the physiological role of apoA-V to evaluate its potential as a target to be up-regulated (e.g. using gene therapy) to decrease plasma triglyceride (TG) levels.

Potential and limitations of AAV-mediated gene delivery

AAV-mediated gene delivery is an efficient tool to induce protein expression in vivo. The expression of transgenes can be induced in several tissues of choice and at a desired level. In practice, obviously, there are limitations to the above-mentioned parameters. For example, AAV is a small virus with a limited packaging capacity (generally considered 4.7 kb, although efficient incorporation of slightly larger genomes has been described1), eliminating the possibility to express large proteins using this viral vector. In addition, not all tissue types are efficiently transduced by AAV and there are limitations to the maximum expression levels that can be obtained. Whereas the packaging size can not be altered, tissue specificity and especially expression levels can be optimized as we show in chapter 3. We demonstrated that expression levels can be strongly elevated by optimizing the transgenic cassette that drives gene expression, e.g. by using tissue-specific promoters and self-complementary (sc) vectors. Additionally, the use of tissue-specific promoters prevents ectopic expression thereby omitting for example expression in antigen-presenting cells which could mount an immune response. Although tissue-specific promoters can strongly increase transgene expression, their optimized use depends on optimized delivery to the desired target cell. To this end, one has the choice out of different AAV serotypes with different tissue tropisms.2 As we showed in chapter 3, careful selection of AAV serotype greatly contributes to improving expression in the selected target tissue. In our case, aiming to optimize hepatic transgene expression, AAV8 was shown to outperform AAV1, 2, and 5. To efficiently target other tissues for
different applications, it is therefore recommended to perform a careful evaluation of available serotypes (9 serotypes are currently routinely used in experimental studies but over 100 new AAV sequences have recently been discovered\textsuperscript{3}) in combination with tissue-specific promoters in order to optimize expression. Direct administration of the viral vector into the target tissue might be an alternative option to direct tissue-targeting of the employed AAV vector. An example of this is the injection of AAV1 into skeletal muscle of LPL deficient mice to express LPL and reduce plasma TG levels (as used in chapter 7 and previously described by Ross and colleagues\textsuperscript{3}). In fact, this approach also proved effective in reducing plasma TG levels in humans.\textsuperscript{4}

Ongoing optimization of AAV vectors will undoubtedly further increase its experimental use to express proteins and study the functional consequences. For example, compared to transgenic animal models, AAV strategies offer advantages in terms of easier application in different animal models and greater flexibility in timing and level of transgene expression.

The clinical use of AAV and gene therapy as a whole is still in its infancy. Despite the excellent safety profile of AAV vectors, their increased efficacy and specificity, and promising proof-of-principle studies, the outcome of clinical trials with AAV have so far not fulfilled the expectations.\textsuperscript{5} Expression levels in reported studies were in general lower as was expected from animal studies and the duration of expression was in general short-lived. The central limiting obstacle in these studies seems to be a pre-existing immunity against AAV capsid proteins, which appears to be present in the majority of the human population.\textsuperscript{6} Before progression into more general clinical use of AAV, this obstacle obviously needs to be overcome. However, promising efforts are currently undertaken to meet these challenges by using improved/different AAV vectors that avoid recognition by pre-existing immunity\textsuperscript{7} or the use of short-term immune-suppressing regimes that block the response to capsid proteins until they are completely cleared from transduced cells (capsids are not encoded in the vector and are therefore only transiently present).\textsuperscript{8} Given the persisting need to develop treatment strategies for serious monogenetic disorders that can not be treated using conventional medicine, it is conceivable that these issues will eventually be overcome. Based on its impressive potential in animal studies (e.g. as shown in this thesis), and its excellent safety profile\textsuperscript{5,9}, AAV is one of the most promising candidates to be used in the clinic. AAV therapies are likely to be employed for untreatable monogenetic disorders with direct life-threatening effects. However, if proven safe and effective, their use may be expanded to a more general use, e.g. to
express therapeutic proteins such as apoA-I or apoA-V, to increase HDL and decrease TG levels, respectively.

**ApoA-I as a (gene) therapeutic target**

ApoA-I has properties that make it an excellent model molecule to study AAV-mediated expression in the liver (chapter 3). Reasons for its suitability as a model molecule include its small size (243 amino acids), the fact that the liver is an endogenous production site and that apoA-I is secreted into the circulation which makes plasma measurements possible. However, apoA-I has also a large therapeutic potential, most notably because of its anti-atherogenic properties through its involvement in the transport of atherogenic cholesterol to the liver.\(^{10}\) Besides the strong association of low apoA-I levels with an increased risk of coronary artery disease (CAD) in epidemiological studies\(^ {11}\), overexpression of apoA-I in animal models has also been shown to reduce the progression of atherosclerosis.\(^ {12-14}\) Hence, reducing atherosclerosis progression will be the primary objective of AAV-hApoA-I as a gene therapeutic clinical tool. Thus, after the observation in chapter 3 that our strategy to increase apoA-I using AAV resulted in increased HDL-cholesterol levels with beneficial functional consequences in apoA-I-/- mice, we examined in chapter 4 whether this strategy would also reduce atherosclerosis progression in a mouse model of diet-induced atherosclerosis (LDLr-/- mouse model). However, despite near-physiological apoA-I levels (for mice) we observed a decrease in HDL-cholesterol and an aggravation of atherosclerosis. A drastic decrease in endogenous murine apoA-I, induced by expressing hApoA-I, actually resulted in a decrease of total apoA-I in the HDL fraction. Thus the presence of endogenous murine apoA-I in the LDLr-/- mouse model prevented answering the question whether AAV-mediated apoA-I expression was indeed anti-atherogenic. Further research in appropriate animal models, e.g. atherosclerosis models without endogenous apoA-I, will be needed to answer this question. Based on the literature, however, it remains very conceivable that increasing apoA-I will be beneficial in terms of atherosclerosis progression.\(^ {12-14}\) Of note, the fact that inducing expression of hApoA-I decreased endogenous murine apoA-I levels has no obvious implications for potential use in humans. In Chapter 5 we set out to distinguish between the various anti-atherogenic properties of apoA-I. Expressing hApoA-I in an animal model in which apoA-I could not exert anti-atherogenic effects through induction of cholesterol efflux, i.e. mice
having received a bone marrow transplantation with ABCA1−/− cells, would allow evaluating the anti-atherogenic potential of other properties of apoA-I. Unexpectedly, the control groups treated with AAV-GFP died in this study of apparent hepato-toxicity. Although the reason for this is currently unclear, it appears that the particular combination of high GFP expression and irradiation is involved. This because AAV-mediated GFP expression in non-irradiated mice as well as apoA-I expression in irradiated mice caused no apparent problems. Thus, although remaining a very promising experimental set-up, we were not able to draw firm conclusions from this experiment with regard to the potential of apoA-I to protect against atherosclerosis beyond its role in macrophage-specific cholesterol efflux. Based on literature, however, apoA-I remains a very interesting and promising therapeutic target.10,15,16

ApoA-V as a (gene) therapeutic target

After optimization of AAV-mediated hepatic expression, apoA-V was considered as a second promising target. Based on murine knock-out and overexpression models, apoA-V was shown to play a key role in the hydrolysis of plasma TG.17-19 Given the association of increased TG levels with increased risk of CAD20, it could therefore be hypothesized that increased plasma apoA-V levels would be associated with a decreased risk of CAD and that increasing apoA-V might be an anti-atherogenic therapeutic strategy. However, regarding the association of apoA-V with risk of CAD, human data based on common genetic APOA5 variation were contradictory and no plasma apoA-V protein data were available at the time. Thus, before developing AAV apoA-V gene therapeutic approaches, we first examined whether plasma apoA-V was indeed associated with a reduced risk of CAD by measuring apoA-V plasma levels (using an in-home developed ELISA) in a large prospective epidemiological study. In this study, we examined plasma apoA-V levels and APOA5 gene variation in 1000 individuals who during follow-up suffered from CAD and 2000 matched controls who did not (the EPIC-Norfolk cohort; chapter 6). Unexpectedly, plasma apoA-V levels were positively correlated with plasma TG levels and were not associated with risk of CAD. The finding of a lack of association between plasma apoA-V levels and risk of CAD made apoA-V appear to be a less interesting candidate for AAV-mediated targeting to reduce risk of CAD. On the other hand, the positive correlation between TG and apoA-V that was observed in our human study (chapter 6)
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and by other investigators\textsuperscript{21-24} was intriguing, because initial murine studies seemed to indicate a direct role of apoA-V in lowering plasma TG levels.\textsuperscript{17-19} To gain further insight into the relationship between plasma TG and apoA-V, we therefore studied different mouse strains. In chapter 7 we show that the positive correlation between plasma apoA-V levels and plasma TG is also present in mice. Moreover, reducing plasma TG levels in LPL deficient mice by using LPL gene therapy resulted in a decrease in plasma apoA-V. The general notion therefore seems to be that, despite its involvement in improving LPL-mediated lipolysis, variation in plasma apoA-V levels appears to follow fluctuations in TG levels and not vice versa, although kinetic studies are needed to verify this. It is of note, however, that the role of apoA-V in TG hydrolysis may be different when studied under non-physiological overexpression conditions. In situations of marked apoA-V overexpression, as shown in chapter 8, apoA-V appears to be a main regulator of plasma TG levels by virtue of its intrinsic TG-lowering capacity. Since lowering TG levels is associated with a decreased risk of CAD\textsuperscript{20}, a strategy to lower TG by increasing plasma apoA-V levels therefore remains promising. In line, Mansouri and colleagues recently showed that expressing apoA-V in an atherosclerotic mouse model not only lowered plasma TG levels but also reduced atherosclerosis.\textsuperscript{25} In the light of the current metabolic syndrome epidemic\textsuperscript{26}, in which elevated TG levels are a central factor, apoA-V can thus be reconsidered as an interesting therapeutic target; a target that needs up-regulation which can be realized through a gene therapeutic approach.

General conclusions

In this thesis, we show that systemic administration of AAV in mice is an efficient tool to induce long-term expression of therapeutic proteins at high levels by the liver. These features combined with the excellent safety profile make AAV a vector of choice to modulate lipid metabolism by expressing e.g. apoA-I and apoA-V. The studies that were aimed at demonstrating the beneficial effects of apoA-I overexpression have been hampered by unfortunate choices of experimental animal models which revealed unexpected physiological changes. Despite the drawbacks that we experienced, we remain with stating that overexpressing apoA-I is a promising strategy to treat a low HDL-phenotype. Although our initial studies regarding the relationship between apoA-V, plasma TG levels and risk of CAD provided little enthusiasm for apoA-V as a potential target,
recent understanding of the role of apoA-V might change this perspective. Overexpression of apoA-V does lead to reduced plasma TG levels, as we also show in this thesis, and should be reconsidered as a therapeutic target to decrease plasma TG levels. Taken together, the potential of AAV as a vector to express therapeutic proteins such as apoA-I and apoA-V is high and continuing development might lead to a useful addition to the future clinical repertoire.

References


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