Personalized treatment for von Willebrand disease by RNA-targeted therapies

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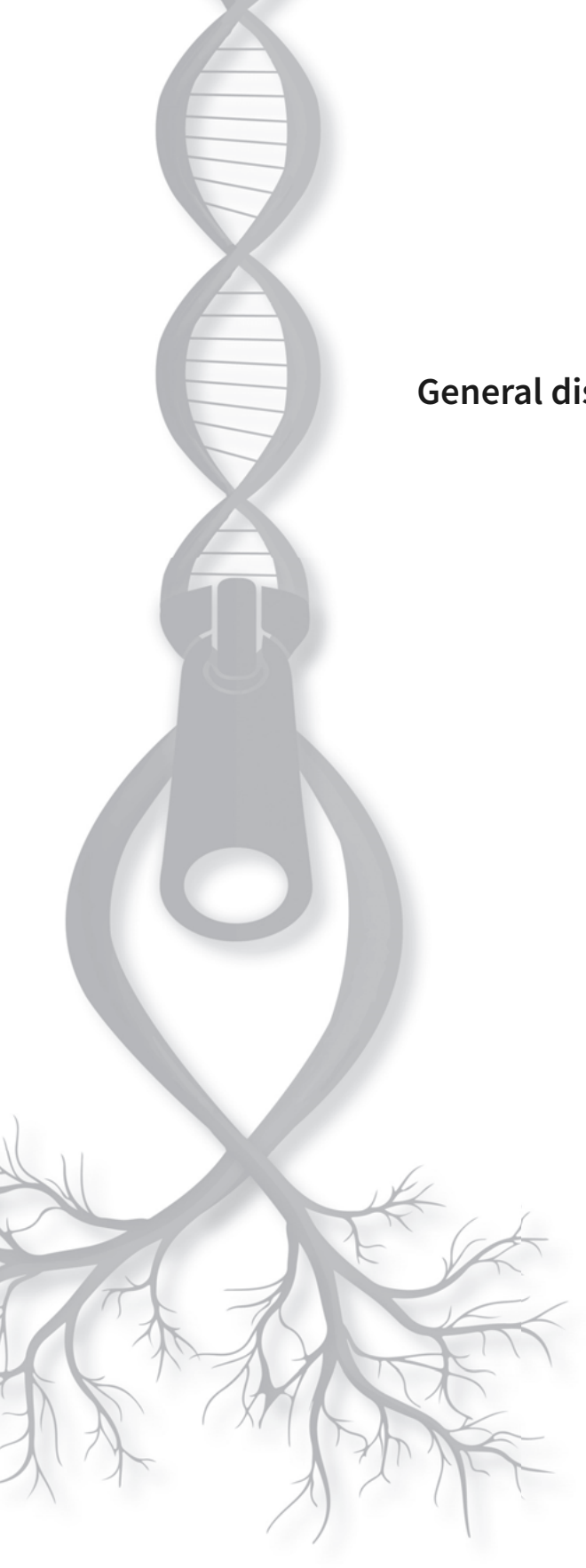

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General discussion and perspectives

This thesis described the proof of concept of allele-specific siRNA-mediated inhibition of dominant negative von Willebrand factor (*VWF*) alleles as a potential therapeutic approach for dominant negative von Willebrand disease (VWD). The main aim was to show that allele-specific siRNAs can efficiently inhibit the production of a mutant *VWF* allele, with no or minor inhibition of the wild type allele, and to show that this positively affects VWF function and VWD phenotypes. The potential of this approach was demonstrated in this thesis *in vitro* in Human Embryonic Kidney 293 cells (HEK293; Chapter 4), *ex vivo* in endothelial colony forming cells (ECFCs; Chapter 7) and *in vivo* in a VWD mouse model (Chapter 8). Also, the applicability of ECFCs as a valid model for VWD has been described in this thesis (Chapters 5 and 6).

A new treatment approach for VWD

Reasoning behind an alternative treatment strategy for VWD

VWD is caused by qualitative or quantitative defects in VWF.[1,2] VWF is known as an important player in hemostasis, where VWF attracts platelets to sites of vascular damage, but also chaperones coagulation factor VIII in the circulation.[3,4] Current treatment options for VWD focus on increasing the levels of VWF in plasma, either by administration of DDAVP or VWF-containing concentrates.[5,6] Both treatment options are generally sufficient to stop or prevent bleeding after trauma or before a planned surgery. However, both DDAVP and VWF-containing concentrates do not address the source of the problem: production of mutant VWF subunits. The production and, subsequently secretion of mutant VWF, may be problematic in a subgroup of VWD patients. Especially these patients have a clinical unmet need and would benefit from a new treatment strategy that blocks the production of mutant VWF only.

The first group of patients with a clear clinical unmet need are VWD type 2B patients. VWD type 2B is caused by dominant negative mutations in the A1 domain of VWF.[2] These mutations result in a conformational change of the A1 domain allowing spontaneous platelet binding, even when VWF is in its closed conformation.[7-9] Because of the enhanced platelet binding, but also because of increased clearance of the VWF-platelet complex, patients develop variable degrees of thrombocytopenia.[10] Treatment of VWD type 2B with DDAVP results in an increased concentration of circulating mutant VWF that captures circulating platelets and induces a further decrease in platelet count.[11,12] Whereas, the VWF present in VWF-containing concentrates will perform normal hemostasis, but will not prevent the removal of platelets by mutant endogenous VWF. Stimuli that are released in certain stress situations, for example during infection, pregnancy or surgery, lead to an increased release of mutant VWF that may induce a deep thrombocytopenia with bleeding as a consequence.[10,13-16] In case of bleeding, patients may need platelet transfusion in addition to VWF-containing concentrates.[13]

A second group of VWD patients that would benefit from a new treatment strategy, are patients that develop severe intestinal bleeding, mainly caused by angiodysplasia (vascular malformations). This diathesis is more common in VWD patients than in the normal population.[17,18] Especially VWD patients with a complete absence of VWF or VWD patients lacking high molecular weight (HMW) VWF multimers have a higher chance of developing gastrointestinal bleeding.[18-20] Severe blood loss from the gastrointestinal tract may result in decreased hemoglobin which sometimes require transfusion of packed red blood cells.[21,22] In the past decade, it became evident that VWF plays a role in angiogenesis, the formation of new blood vessels, which explained the increased patient population that suffer from these severe intestinal bleeding.[23] However, the exact mechanism on how VWF affects angiogenesis and why especially patients that lack HMW VWF are prone to this diathesis remains to be elucidated. Bleeding from the gastrointestinal tract are not easily resolved by on demand treatment with VWF-containing concentrates.[22,24] Probably because the VWF-containing concentrates do not target the source of the problem: deficient or dysfunctional intracellular VWF and long-term exposure to mutant VWF. Long-term prophylaxis of VWF-containing concentrates does reduce the frequency of gastrointestinal bleeding, but comes along with the burden of frequent injections and a reduced quality of life.[21,25-27] A recent report on a small number of VWD patients suggests long-term use of Lenalidomide, a thalidomide analog with anti-angiogenic properties.[28] Although this proved relative successful in this group of patients, it again does not address the causative factor: mutant VWF. Also, another study in which thalidomide was used did not show any effects in the treatment of gastrointestinal bleeding.[20] In short, there is no clear-cut solution for treatment of angiodysplasia-related gastrointestinal bleeding.

Above, two groups of patients are described with a clear clinical unmet need. However, long-term correction of VWF is expected to also benefit patients without a clinical unmet need. Although the current treatment modalities are able to prevent or stop bleeding during surgery or after trauma, patients continuously live with the knowledge that bleeding can suddenly occur. Also, an ongoing bleed affects the patients daily life, and may require a hospital visit. This results in an overall reduced quality of life in VWD patients.[29-32]

Expected effects of allele-specific inhibition of mutant VWF for VWD patients

The expected effects of allele-specific inhibition of mutant *VWF* depends per type of VWD. Allele-specific inhibition of mutant *VWF* to correct VWD will only correct VWD caused by heterozygous dominant negative mutations, i.e. VWD types 1, 2A, 2B and 2M.[33] Both VWD type 2N and VWD type 3 result from homozygous mutations and will therefore not be corrected using our approach of allele-specific *VWF* inhibition.[34]

VWD type 1 is associated with decreased VWF plasma concentration that is the consequence of decreased VWF production, defective VWF secretion or an increased clearance of VWF.[2] A decreased VWF production is often the result of a heterozygous null allele, and cannot be corrected by allele-specific inhibition of mutant *VWF*.[35] Allele-specific inhibition of *VWF* in VWD type 1 patients with decreased VWF secretion is expected to improve the secretion of VWF from the endothelial cells.[36-38] When the secretion defect is severe, correction of this secretion defect by allele-specific siRNAs might even result in an overall increase in circulating VWF, even though the overall production of VWF is roughly halved after inhibition of the mutant allele. Allele-specific inhibition of mutant *VWF* in VWD type 1 patients with enhanced VWF clearance is expected to increase the survival of VWF.[39] When the clearance effect of a dominant negative mutation is strong, downregulation of the dominant negative allele might increase the overall VWF plasma concentration, irrespective of the reduced VWF production after siRNA inhibition.

VWD type 2A is associated with reduced HMW VWF, caused by either an intracellular multimerization defect or enhanced cleavage of VWF by ADAMTS13.[2,40,41] Inhibition of mutant VWF in both situations is expected to increase the concentration of the largest VWF multimers in the circulation. Since especially HMW VWF has the highest hemostatic activity, it is expected that downregulation of mutant VWF in VWD type 2A results in improved hemostatic function of VWF and amelioration of the patient's bleeding phenotype. In this thesis, we indeed proved in HEK293 cells (Chapter 4) and in ECFCs (Chapter 7) that inhibition of mutant VWF results in an increase in HMW VWF for two different mutations associated with an intracellular multimerization defect.[42] Whether this also results in improved hemostatic function remains to be answered from preclinical VWD models. Angiodysplasia-related intestinal bleeding are also common among VWD type 2A patients. Downregulation of the expression of mutant VWF is expected to have two advantages. First, it is thought to positively affect the process of angiogenesis and thereby reducing the chance of developing angiodysplasia. Second, inhibition of mutant VWF increases the hemostatic function of VWF and thus reduce the possibility to develop severe bleeding. It is however expected that a continuous correction of VWF is required to correct for the defective angiogenesis. Dysfunctional VWF does not only result in intestinal angiodysplasia, but also in vascular malformations elsewhere, like in the nailfold.[43] Long-term correction of dysfunctional VWF is therefore also expected to correct for vascular malformations at other sides then the intestine.

VWD type 2B is associated with reduced VWF survival, a decrease in HMW VWF, and variable degrees of thrombocytopenia.[7,44] The reduced VWF survival in VWD type 2B is suggested to be (at least partly) caused by an increased binding affinity of mutant VWF to the clearance receptor lipoprotein receptor-related protein 1 (LRP1) on macrophages.[45,46] Inhibition of mutant VWF is expected to result in decreased binding affinity of VWF to LRP1, thereby increasing the

survival of VWF. The decrease in HMW VWF is the result of increased clearance of the larger VWF multimers, but also by enhanced sensitivity of mutant VWF to proteolysis by ADAMTS13.[47] Inhibition of mutant VWF is expected to correct for both aspects. The thrombocytopenia in VWD type 2B is the consequence of increased clearance of the VWF-platelet complex, but also by modified megakaryocytopoiesis and affected platelet production.[46,48,49] Inhibition of mutant VWF is expected to reduce the binding of VWF to platelets, thereby reducing the elimination of circulating platelets. Indeed, in this thesis we show that inhibition of mutant VWF in a heterozygous VWD type 2B mouse model results in correction of decreased platelet counts (Chapter 8). Besides low platelet counts in VWD type 2B, also enlarged platelets and platelet aggregates are observed in VWD type 2B patients.[49] Furthermore, binding of mutant VWF to the platelets glycoprotein Ibα (GPIbα) receptor was recently shown to affect platelet function by altered platelet signaling.[50,51] Inhibition of mutant VWF is therefore expected to increase platelet function as well. siRNA-targeting to the megakaryocytes and platelets is yet impossible, and therefore we do not expect to correct for defects caused by mutant VWF that is produced in the megakaryocytes/platelets. It is, however, unclear what the relative effects of megakaryocytes/platelet produced mutant VWF on platelet function and production are. We do know that $VWF^{-/-}$ mice expressing the VWD type 2B mutation mVWF p.Val1316Met after hydrodynamic injection show an increase in platelet size, while these mice do not express platelet mVWF.[52] We were able to reduce the platelet size after allele-specific inhibition of the production of mVWF p.Val1316Met (Chapter 8), which suggests that mainly circulating VWF, and not platelet VWF, is responsible for the affected platelet size and production.

VWD type 2M is caused by heterozygous mutations in the A1 domain of VWF that decreases the binding affinity of VWF to platelets GPIbα or collagen.[2] Inhibition of mutant VWF by allele-specific siRNAs are likely to improve the VWF activity. However, since less VWF will be produced and secreted, it is uncertain whether this will also result in increased platelet binding and improvements in the hemostatic function of VWF. Flow experiments on the platelet-binding activity in ECFCs derived from a VWD type 2M patient treated with allele-specific siRNA might reveal whether inhibition of mutant VWF in VWD type 2M improves the platelet binding capacity.

Besides the role of VWF in hemostasis, several additional roles for VWF have been elucidated in the past years. Examples are roles of VWF in inflammation, wound healing, and smooth muscle cell proliferation.[53-55] Although the consequences of mutant VWF on these processes are yet unknown, it is likely that it somehow affects VWD patients. Therefore, it is expected that inhibition of mutant VWF will not only improve the hemostatic function of VWF, but also roles of VWF beyond hemostasis.

Clinical application of allele-specific siRNAs

How siRNA-mediated therapeutics for VWD might be implemented in the clinic depends on the indication and the duration of siRNA efficacy. So far, there is no clinical data on the efficacy of siRNAs in the endothelium. However, recent data shows long-term efficacy of siRNAs in the human liver and in mouse endothelium.[56-58] Even though merely speculative, we assume a long-term siRNA efficacy when we envision the application of siRNA therapeutics in the clinic.

The most important indication for RNA therapeutics in VWD are surgery or dental procedures. An injection of allele-specific siRNA prior to a planned surgery is expected to prevent excessive bleeding during surgery, but could also help in the healing process after surgery.[54] Injection of an allele-specific *VWF* siRNA might be accompanied by administration of DDAVP. This would result in a short-term increase of fully functional VWF, enough to cover a planned intervention. In VWD type 2B patients, DDAVP could then safely be administered without the fear of provoking thrombocytopenia.[12] The approach would also allow the use of DDAVP in patients that are normally unresponsive to DDAVP, for example patients with a severe secretion defect.[59] These patients otherwise require replacement therapy.

Another important indication, also discussed above, are angiodysplasia-related gastrointestinal bleeding. Since administration of VWF-containing concentrates is usually not sufficient to stop bleeding and only long-term prophylaxis reduces the change of developing a gastrointestinal bleed[21,24], it is expected that only long-term correction of VWF by allele-specific siRNAs would reduce the risk of developing angiodysplasia-related gastrointestinal bleeding.

When a VWD patient develops a bleed that requires replacement therapy, the patient is generally referred to a hospital for an injection with VWF-containing concentrates. In the Netherlands, patients are referred to specific hemophilia treatment centers, which are in a relative short distance for most people.[60] However, a visit to a hospital could be more complicated when living in a rural area or when on holidays. Long-term correction of VWF by allele-specific siRNAs might be a solution for those patients that normally would require regular monitoring but live in more rural areas, but also when a patient plans a holiday to a less accessible location.

RNA therapeutics

We currently live at an exciting time with respect to siRNA therapeutics. In 2018, exactly 20 years after the first publication of RNA interference by Mello and Fire, the first siRNA drug (Patisiran) was approved by the U.S. Food and Drug administration.[56] Patisiran is an siRNA, encapsulated

in a lipid nanoparticle, that effectively target transthyretin (*TTR*) in the liver and ameliorates hereditary transthyretin amyloidosis. Clinical trials show effective TTR knockdown using an every-three week dosing regimen. Besides Patisiran, many other siRNA drugs have made it to clinical trials, and new approvals are to be expected in the coming years.[61] The most important results from these clinical trials for our work is the long-term efficacy of siRNAs after a single dose. For example, a single dose of Fitusiran, an N-acetylgalactosamine (GalNAC)-conjugated siRNA against antithrombin, anticipated as an alternative therapy for prevention of bleeding in hemophilia A and B patients, resulted in strong antithrombin inhibition for at least 30 days.[62] And even more remarkably, a single dose of Inclisiran, a GalNAC-conjugated siRNA against protease proprotein convertase subtilisin/kexin type 9 (*PCSK9*), anticipated for use in familial hypercholesterolemia patients, resulted in a very strong knockdown of *PCSK9*.[63] With even after 180 days, a PCSK9 knockdown that ranged between 47.9 and 59.3%.[63] These are a few examples of the current successes in siRNA therapeutics and highlights the potential of this class of drugs in the treatment of genetic disorders, like VWD.

Allele-specific siRNA target selection for diseases caused by dominant negative mutations

siRNAs degrade mRNA sequences based on full complementarity. The cleavage ability of an siRNA might be disrupted when a mismatch is present between the siRNA and the mRNA.[64] This feature allows the design of allele-specific siRNAs: siRNAs that inhibit the production of one allele of a gene without affecting the other allele. Allele-specific inhibition requires nucleotide variations between two alleles of a gene. These variations can be a heterozygous dominant negative mutation, but also heterozygous single-nucleotide polymorphisms (SNPs) that are linked to the dominant negative mutation.[65-68] The use of a heterozygous dominant negative mutation as a target for an allele-specific siRNA is an ideal approach for diseases that result from a single mutation. However, VWD, and also many other diseases, result from many different mutations.[2] Therefore, to develop a treatment approach for dominant negative VWD that is to be applied to a large patient population and not only to individual patients, we have chosen for a SNP-based approach. SNPs are nucleotide substitutions the are present in the human genome.[69] Most of these SNPs are harmless and are not associated with disease. Also, some SNPs are common, meaning that in a population many people are heterozygous for these SNPs. We have made use of very common SNPs in the coding sequence of *VWF* and selected four SNPs for which about 74 percent of the Caucasian population is heterozygous.[42] The percentage of the population that is heterozygous for at least one of the four SNPs was only calculated for the Caucasian population, however also in other populations investigated in the 1000 genomes project, a high minor allele frequency was found for the same four SNPs.[69] An important remark is that two of the four selected SNPs, c.2365A|G and c.2385C|T, have been associated with VWF levels.[70-72] However, since the effects of the SNPs are minimal in

comparison to the effects of the dominant negative mutations that cause VWD, it is expected that allele-specific inhibition of these SNPs will have minor effect on the correction of the phenotype.

Design of allele-specific siRNAs

For the studies described in this thesis, siRNAs have been designed against both alleles of the four selected SNPs. In our studies, 21 nucleotide siRNAs with a dTdT overhang at the 3' end of the sense strand were used. Therefore, 21 siRNA designs are possible that include the SNP in the antisense strand. Ideally, all of these siRNAs are tested to identify the most efficient and allele-specific siRNA candidate. This is however a costly procedure. Fortunately, the twenty year experience in siRNA design resulted in algorithms that may predict the efficiency of siRNAs.[73,74] Therefore, only the three siRNAs with the highest predicted efficacy were designed and tested per SNP target (siRNA designs were done by Life Technologies).[42] Besides the requirement that the designed siRNAs should be efficient, the siRNAs should also be specific for its SNP target. Over the past two decades, many studies applied allele-specific inhibition and used SNPs or mutations to discriminate between two alleles, of which some examples can be observed in the following references.[65,66,75-78] Based on these studies, many thoughts have been generated on what factors are important in the discriminatory effect of siRNAs. Recently, also an online algorithm was published that should help in the design of allele-specific siRNAs (http://crdd.osdd.net/servers/aspsirna/index.php).[79] General ideas of discriminatory elements of allele-specific siRNAs are: (1) highest discrimination is observed when the mismatch is located in the center of the siRNA[80,81], (2) the mismatch should not be located in the seed sequence[80], (3) and better discrimination is observed when the mismatch involves a purine/purine mismatch between the siRNA and the target mRNA (i.e. adenine/guanine mismatch).[80,82] Interestingly, although most siRNAs that proved effective in our studies had their mismatch located in the center of the siRNA, we also observed effective allele-specific inhibition for siRNAs where the mismatch was not located in the center. Furthermore, none of our siRNA/mRNA interactions involved a purine/purine mismatch, nevertheless we observed a very good discrimination for several of our designed siRNAs. Especially siRNAs that target either *VWF* c.1451A or *VWF* c.1451G were very effective, while they create a pyrimidine/purine mismatch between the siRNA and mRNA sequence. Differences between our observations and previous observations may result from differences in the chemical modification of the siRNAs. We used siRNAs with a locked nucleic acid (LNA) modification that is known to increase the specificity of the siRNAs.[83] Furthermore, every mRNA sequence has a different secondary structure, which is likely to affect the binding of an siRNA to the mRNA. It is therefore difficult to predict the specificity of an allele-specific siRNA and it is suggested to test multiple allele-specific siRNA candidates for their effectiveness and not fully rely on prediction tools.

Previous work on allele-specific siRNAs

In the past two decades, several *in vitro, ex vivo* and *in vivo* studies have been performed on the applicability of allele-specific siRNA-mediated inhibition of mutant alleles to improve disease phenotypes, and many with great success.[65,66,75-78] However, only one has made it to a clinical trial (NCT00716014), and this trial only involved one patient.[84] Although the trial was relative successful, no further studies have been reported on this treatment. Speculation can be done on the reason of this lack in progress. First of all, further development of drugs and start of clinical trials are expensive and will in general require a sponsor, most often a company. But, allele-specific inhibition of mutant alleles is a highly personalized treatment, and is only applicable to patients that harbor the specific mutation/SNP where the siRNA was designed for. For example, as for the only clinical trial that has been performed so far, the specific siRNA was applicable to only three patients.[84] The costs for further development will be excessive, and will never be reimbursed. Another complication of allele-specific siRNAs is that appropriate animal models are often unavailable to perform preclinical studies on. The chance that the sequence around a SNP or mutation is similar in mice and monkeys than it is in humans is small. Therefore, it is difficult to study the specific effects of siRNA-mediated inhibition in a preclinical model, unless humanized disease models are generated and used.[85,86] The last reason is the difficulty in the delivery of siRNAs to specific cell types. Naked unmodified siRNAs are cleared within 5 minutes from the body by the kidney, liver and spleen, and delivery vehicles are needed to get the siRNA to the desired cell type.[87,88] Targeting the hepatocytes of the liver is known to be relatively easy using lipid nanoparticles, or GalNAC conjugates.[88,89] However, the development of delivery vehicles to extra-hepatic organs remains challenging, and it is only for the last few years that effective delivery vehicles have been described and tested in preclinical models.[57,58] It is therefore expected that clinical trials using these delivery vehicles are not far from happening.

Extra-hepatic delivery of siRNAs

Most successes of siRNA therapeutics so far use liver-targeted siRNAs. Hepatic siRNA targeting is relatively easy and is mainly achieved by complexation of siRNAs in liposomes, or conjugation of an internalizing peptide to the siRNA. Therefore, for the *in vivo* proof of concept of allele-specific inhibition of mutant *VWF* as a potential treatment approach for VWD, we choose a VWD mouse model with liver-expressed VWF (Chapter 8). Using Invivofectamine (Thermo Fisher Scientific, Carlsbad, CA, USA), a commercial available liposomal formula, we successfully inhibited VWF in the liver, and proved that allele-specific inhibition of mutant *VWF* in a heterozygous VWD type 2B mouse model improves the disease phenotype. Next, it is important to translate these results to a VWD model where mutant VWF is expressed in a physiological fashion, i.e. a mouse model with endothelial produced mutant VWF. The first challenge to overcome: how to get the siRNA into the endothelium. Fortunately, recent

developments show promising results in this respect. The most successful approaches that have been described use cationic lipids, polymeric nanoparticles or lipid-conjugated siRNAs.

The first studies that successfully achieved endothelial targeting by siRNAs used cationic lipids.[58,90-96] siRNAs are complexed in cationic lipids by electrostatic interactions between the negative siRNA and the cationic lipid.[58] An example of a cationic lipid is ActuFECT01, which showed good distribution to the endothelium.[91-95] ActuFECT01 has been used in the lipoplex formulation of Atu027, a lipoplex containing an siRNA that targets protein kinase N3. Atu027 even made it to a phase I clinical trial (NCT00938574) in the treatment of solid tumors.[97] A second phase I/II trial (NCT01808638) has been completed in March 2016, however no data have been presented on the results thus far. Although effective knockdown of endothelial genes was achieved, repeated dosing was needed to achieve robust *in vivo* knockdown and the siRNA also internalized in resident macrophages.[96] Changes in the composition of lipoplexes containing the cationic lipid ActuFECT01, resulted in the development of a novel lipoplex formulation: DACC.[58] DACC showed especially in the lung vasculature robust knockdown of several endothelial genes, and importantly, one dose of siRNA against *Tie2* resulted in *Tie2* knockdown for up to 21 days.[58] However, since the first report in 2014, no new data have been published on the use of DACC. The mechanism of internalization of cationic lipids is so far unknown. Fehring *et al* speculate that electrostatic interactions between the positively charged lipoplex and the negatively charged endothelium might result in internalization of these lipoplexes into the endothelium.[58]

A second class of endothelial targeted compounds are polymeric nanoparticles. Instead of cationic lipids, polymer lipids are used and siRNAs are complexed within a polymeric nanoparticle by multivalent interactions. Dahlman *et al* describe the formulation of 7C1, an ionizable low-molecular weight polymeric nanoparticles, and show that 7C1 forms stable siRNA/lipid particles.[57] 7C1 is especially effective in targeting lung endothelium, without targeting hepatocytes or immune cells. Robust knockdown of several endothelial genes was proven and siRNA-mediated inhibition of *ICAM2* was even retained for a period of 21 days after a single siRNA injection.[57] Follow-up studies showed effective endothelial gene knockdown by siRNAs encapsulated in 7C1 in lung endothelium, but also in tumor endothelium.[98-100] Also, effective delivery of microRNAs or antisense oligonucleotides against microRNAs (antimiR) was proven by the use of 7C1.[101,102] Furthermore, simultaneous inhibition of 5 genes was even feasible using multiplex 7C1 complexation.[103,104] Translation of the use of 7C1 from mouse models to non-human primates was recently reported.[105] Non-human primates were injected with an siRNA against *Tie2* and a strong downregulation of *Tie2* was observed on RNA level in the lung and the heart. This, however, did not result in reduced Tie2 protein levels. However, only three animals were used in the treatment group and the animals were sacrificed 48 hours after treatment, which might have been too early to observe effects on protein level. Most

importantly, no acute signs of toxicity were found after siRNA injection in the primates.[105]

Both cationic lipids and polymeric nanoparticles show highest internalizing capability in the lung endothelium. For our studies this is highly advantageous, since the lung endothelium is responsible for a large part of the VWF production.[106] The mechanism why especially the lung endothelium is targeted remains unknown.

The last strategy of endothelial targeting discussed here is lipid-conjugated siRNAs.[87] A recent study by Biscans *et al* show the biodistribution of a diverse panel of lipid-conjugated siRNAs to extra-hepatic tissues.[107] Several conjugates show, amongst others, distribution to the lungs and heart. This study did not look specifically into endothelial genes, and the potential of downregulation of genes in the lung and liver were yet limited. However, knowledge of the type of lipid conjugate that is internalized in the lungs and heart is a start for further research to the engineering of lipid conjugates that are internalized by endothelial cells. A limitation of lipid conjugates is that they do not specifically target a tissue and large parts of the injected lipid is cleared by the liver, kidney and spleen.[107] This increases the chance of off-target effects, however since VWF is only synthesized in the endothelium and megakaryocytes, this is unlikely to be a serious problem.

Altogether, serious progress has been made in the development of compounds to deliver siRNAs into the endothelium. Especially cationic lipids and polymeric nanoparticles show robust and long-term inhibition of endothelially genes *in vivo*, especially in the lung vasculature. These results are promising for future studies on RNA-targeted therapies for VWD.

Improvements in siRNA modifications to make them more effective

The siRNAs used in studies reported in this thesis are chemically modified by LNA modifications.[83] LNA is a modification in which the 2' oxygen and 4' carbon of the ribose are linked through an extra bridge. This extra bridge enforces the ribose in the 3'-endo conformation, which gives it a higher affinity to its target. It is also known for its strong ability to discriminate between nucleotides.[108] Besides LNA modifications, many more functional modifications were proven effective in siRNA design. Examples of widely used modifications are 2'-O-methyl, or 2'-fluoro modifications of the ribose ring. Testing siRNAs with different modifications might even lead to the improvements in the functionality of allele-specific siRNA candidates.[109]

Alternatives to siRNA therapeutics

As approach to inhibit mutant *VWF* alleles as an alternative treatment approach for dominant negative VWD, we have chosen to use allele-specific siRNAs. Allelic discrimination could, however, also be achieved by different approaches that target either mRNA or DNA. These approaches include the use of amongst others antisense oligonucleotides (AONs), miRNAs, CRISPR/Cas9 or Zinc Finger nucleases.[86,110-112] Application of AONs and Crispr/Cas9 will be discussed below.

AONs are short single-stranded DNA molecules that have the ability to target the pre-mRNA in the nucleus, or the mRNA in the cytoplasm.[113] The mechanism of action of antisense oligonucleotides depends on the modification of the AON sugar ring.[114] Unmodified AONs are rapidly degraded by nucleases.[115] Backbone chemistries are therefore necessary to limit nuclease degradation.[115] Furthermore, sugar ring modifications enhance the binding capacity of the AON to the (pre)-mRNA target.[116] mRNA cleavage after AON targeting is facilitated by RNase H, but this is not possible when all the nucleotide sugar rings are chemically modified. Therefore so-called 'gapmers' have been designed that contain fully modified outer nucleotides, but have an unmodified DNA core of eight to ten nucleotides.[116] This allows strong binding of the AON to the (pre)-mRNA, but may still facilitate RNase H dependent cleavage.[114] RNase H-mediated cleavage can take place both in the cytoplasm and in the nucleus, which allows the use of gapmers to target intronic sites as well.[117] Since many SNPs are located in intronic regions, more targets can be reached by gapmers than by siRNAs. Furthermore, chemically modified AON backbones are more easily taken up by a variety of cells in the body and can therefore be injected without the requirement of a delivery vehicle (naked delivery).[118] It is, however, questionable how much is internalized by the endothelium. Therefore, AON conjugation to an internalizing peptide or complexation of an AON in a lipid or polymeric nanoparticle might nevertheless be necessary for endothelial uptake. An allele-specific gapmer that targets a SNP in the 3' UTR of Huntingtin has made it to an ongoing Phase I/II clinical trial (NCT03225833). Preclinical data using this gapmer in a humanized Huntington's disease mouse model showed persistent AON activity for several weeks.[86] Also other clinical trials using non allele-specific AONs show long-term efficacy.[85,119] It is not possible to predict which of the two are superior, AONs or siRNAs. This can only be identified by experimental investigations. Difficulty with AON testing, however, is that thousands of different AONs with variable lengths and chemistries can be designed per target, and that algorithms that predict the efficacy of AONs are unavailable.[114] This is a costly investigation, and practically impossible to be investigated in an academic setting.

Both siRNAs and AONs target the mRNA or pre-mRNA and the effects they have on protein expression are therefore transient. Repeated dosing is necessary to maintain a sustained correction of the defective protein. In the past years, a tremendous amount of work has been

performed on the CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats) system to modulate the genome.[120] The mechanism of CRISPR/Cas9 is based on a single guide RNA (sgRNA) designed to complement a DNA sequence and that directs Cas9 to the DNA. Cas9 is an endonuclease that induces a double-strand break in the DNA, which is repaired by non-homologous end joining, one of the cell's repair mechanisms. However, non-homologous end joining is an error-prone repair system, and often results in disrupted genetic sequences.[121] When an sgRNA is designed to target a dominant negative mutation or a SNP that is linked to a dominant negative mutation, allele-specific gene disruption may be accomplished.[111,122,123] One requirement is that Cas9 needs a PAM (Protospacer adjacent motif) sequence on the DNA to achieve DNA cleavage.[120] When this sequence is not present around the SNP or mutation, allele-specific CRISPR/Cas is not possible. However, recent investigations in different or modified Cas9 proteins have resulted in Cas9 proteins that can recognize different PAM sequences, thereby increasing the number of Cas9 targets.[124,125] An important and often discussed aspect of CRISPR/Cas9 are off-target effects.[126,127] Since CRISPR/Cas9 alters the DNA, off-target effects may have serious consequences. Good target selection and sgRNA design is therefore a crucial step in developing CRISPR/Cas9 therapeutics. However, allele-specific genome editing is restricted to sites of heterozygous variations, which limits the possibility for specific sgRNA designs and increases the chance of off-target effects. Another difficulty with the CRISPR system is the delivery of both a sgRNA and an mRNA encoding Cas9 or a Cas9 protein into endothelial cells.[128] Viral delivery might be possible, but does not selectively target endothelial cells. Recently, a polymeric nanoparticle was described that was able to deliver both the Cas9 mRNA and an sgRNA into endothelial cells. These results are promising and may have clinical potential.[129]

Altogether, besides siRNAs, several other approaches may successfully be used as alternative treatment approach for dominant negative VWD. Since the effects of siRNAs and AONs are transient, it is thought to be a more safe approach than CRISPR/Cas9 that permanently edits the DNA. Since VWD is not associated with a very low quality of life or a high mortality, permanent correction of VWD by CRISPR/Cas9 might not outweigh the potential risk of off-target effects. On the other hand, developments in CRISPR/Cas9 quickly emerge and it might therefore be possible that safe usage and specific delivery of CRISPR/Cas 9 is feasible in a few years from now.

Cellular and animal models to study VWD

In this thesis, several different cellular and animal models have been used to study the effects of allele-specific siRNAs against *VWF*. First, siRNAs have been tested for their efficacy in VWF overexpressing HEK293 cells.[42] HEK293 cells do not endogenously produce VWF, but the cells

are easily transfected with VWF constructs, resulting in a functional VWF protein. HEK293 are commonly used cells in VWD research, since they are one of the only cells that are able to store VWF in pseudo-WPBs.[130] Many disease-causing mechanisms have thus been revealed by HEK293 cells that were transfected with mutant VWF constructs.[131-133] Since HEK293 cells are easy to transfect, they are also commonly used for the screening of siRNA candidates. To test the efficacy of the allele-specific siRNAs in our study, siRNAs were transfected in HEK293 cells together with two full-length VWF constructs that contained either of a SNP allele. To discriminate between the two gene products on protein level, Myc and HA peptide tags were added to the constructs. This allowed quantification of protein expression of both constructs separately by ELISA. Using this method, we successfully selected a set of siRNAs that discriminate between two *VWF* alleles.[42] A different and often used method to screen for effective siRNAs is to make use of reporter constructs containing a minigene.[134-136] These reporter constructs contain only part of the gene, for example one exon, and a luminescent gene. Cotransfection of the siRNA with two constructs containing either of an allelic variant and a different luminescent gene allows high-throughput screening of siRNA candidates. Although this is a fast approach to screen for siRNA candidates, it does not guarantee that the selected siRNAs are also effective when the full-length cDNA is present. This is probably due to a different secondary mRNA structure of the gene product of the minigene that affects the binding of the siRNA to the mRNA.[137] Although the process is slower, it is therefore suggested to screen the siRNAs in a system containing full-length *VWF* cDNA.

HEK293 cells proved a good model for the selection of siRNA candidates. However, they do not endogenously produce VWF. It was therefore important to translate the results from HEK293 cells to an endothelial cell line. As *ex vivo* endothelial disease model, we have chosen to use endothelial colony forming cells (ECFCs) that were isolated from a VWD patient and several healthy controls. ECFCs have proven a good *ex vivo* endothelial model to study VWD in the past years, and several disease causing mechanisms have been studied using these cells.[38,138-141] Although ECFCs are an interesting cell model to study disease-causing mechanisms, there are also many limitations associated with the use of these cells. First of all, the success rate of obtaining proliferative ECFC clones is low. A recent report stated a success rate of 70-75 percent[142], however in our lab this success rate is somewhat lower and is just below 50 percent (unreported data). The reported success rate may be biased because many reports do not state the number of unsuccessful isolations. Also, when it is possible to isolate ECFCs from a specific donor, there is a high chance that this donor will consistently yield successful ECFC isolations. Repeated isolation from the same donor would increase the success rate of a lab. The success rate from our lab may be lower, since it does not include many repeated isolations. Then, ECFC lines that are successfully isolated show a large variation in morphology, proliferation rate, and VWF-related parameters.[143,144] Work described in this thesis indicate the isolation of sixteen ECFC colonies from six healthy donors and show that all cell lines

show a different cell density when they are at their maximum confluency.[144] This cell density significantly correlated with the VWF production of the cells. Since there is a clear variation observed between ECFC lines, it is impossible to compare two cell lines with each other that have completely different cellular characteristics. Care should therefore be taken to match a patient-derived ECFC line with a control ECFC line with the same cellular characteristics. Healthy control ECFCs have been used in this thesis to study the efficacy of allele-specific siRNAs against *VWF* SNPs. We show a good correlation of the effects of the allele-specific siRNAs in HEK293 cells and in ECFCs. This also validates that full-length VWF overexpressing HEK293 cells is a solid cell model to screen for siRNA candidates. Allele-specific siRNAs have also been tested for their ability to correct a VWD phenotype. In this experimental set-up, the effects of an allele-specific siRNA on a patient-derived ECFC line are compared to the effects seen in the same patient-derived ECFC line transfected with a negative control siRNA. ECFCs are thus used as their own internal control in this situation, and do not have to be compared to other ECFC lines with possible different cellular characteristics. For this thesis, allele-specific siRNAs have been tested on one patient-derived ECFC line. Unfortunately, several attempts to isolate ECFCs from other patients were not successful. Since the success rate of ECFC isolation is low, an alternative approach to obtain patient-specific cells is desirable. An alternative source of endothelial cells are induced pluripotent stem cells (iPSCs) differentiated to endothelial cells.[145,146] iPSCs can be generated from several cell types, amongst others urine, fibroblasts or peripheral blood mononuclear cells, with a much higher success rate than ECFC isolations.[147,148] Especially the increased success rate of obtaining endothelial cells from an easily accessible cell source is highly advantageous. However, the suitability of iPSC-derived endothelial cells as a model for VWD has yet to be proven.

For the *in vivo* proof of principle studies, a VWD mouse model with hepatic VWF expression was used. This well-established mouse model is generated by hydrodynamic injection of *Vwf* cDNA in $VWF^{-/-}$ mice on a C57BL/6J background.[52] Hydrodynamic injection of mutant *Vwf* cDNA have repeatedly proven to result in a phenotype resembling that of VWD patients.[52,149] Heterozygous VWD type 2B mice were generated by injection of both wild type and mutant *Vwf* cDNA. This resulted in a VWD type 2B phenotype resembling that of VWD type 2B patients. Delivery of siRNAs to induce allele-specific inhibition of mutant *VWF* was facilitated by Invivofectamine, a commercially available lipid nanoparticle that efficiently delivers siRNAs to the hepatocytes.[150,151] This proved to be a very successful method for the proof of principle of allele-specific inhibition of *VWF* to correct the VWD type 2B phenotype. However, to become clinically relevant, the effects of allele-specific siRNAs to ameliorate VWD phenotypes should be tested in a more physiological model with endothelial expressed mutant VWF. So far, only two VWD knock-in mouse models have been described.[152,153] One of these VWD knock-in mouse models harbour the mouse VWF p.Val1316Met mutation[152], and it would be of great interest to investigate our siRNA designed to target the mouse VWF p.Val1316Met in

this mouse model using one of the recently developed endothelial delivery vehicles.[57,58] This allows to investigate the efficiency of (allele-specific) siRNAs to inhibit VWF in endothelial cells *in vivo*. Furthermore, it would be possible to test the effects of inhibition of mutant *VWF* on the hemostatic capacity of these mice, by for example a bleeding assay.[152] Unfortunately, these knock-in mouse models do not allow to test the SNP-targeted allele-specific siRNAs. This can only be achieved by (humanized) heterozygous mouse models containing the human SNPs, but also the human sequence around the SNPs.

Concluding remarks

Inhibition of mutant *VWF* alleles by allele-specific siRNAs was proven in this thesis to be a promising strategy to correct for VWD phenotypes. Proof of principle studies were performed in HEK293 cells, ECFCs and a VWD mouse model with hepatic VWF production, and in all of these disease models a correction of VWD phenotypes was observed after transfection or injection of allele-specific siRNAs. The field of RNA therapeutics has made a tremendous progress in the past years with respect to siRNA design and endothelial siRNA delivery. These developments are promising regarding our approach of RNA therapeutics to correct VWD phenotypes. Studies in (humanized) preclinical models should further elucidate the potency of allele-specific siRNAs as alternative treatment approach for dominant negative VWD.

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