Preparing for CADASIL therapy

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Chapter 6

Discussion and future perspectives

The aim of this PhD project was to advance CADASIL therapy development. This involved studies aimed at obtaining *in vivo* proof-of-concept for NOTCH3 cysteine correction, as well as generating outcome measures to be used in future (pre-)clinical trials.

There is a high unmet medical need for CADASIL patients given the impact of the disease on the life of patients and their family members, and the lack of a treatment that can cure or delay CADASIL. Although patients may receive symptomatic treatment, this does not avert the development of stroke or vascular dementia. Therapies currently under development for CADASIL are aimed at preventing or halting $NOTCH3^{ECD}$ aggregation or mural cell degeneration.[1–4] The optimal time to initiate treatment is probably as early in the disease course as possible, but at least before patients have irreversible brain damage. Young adult patients may already have some $NOTCH3^{ECD}$ aggregation and WMH, but they rarely have clinical manifest CADASIL. Pre-symptomatic patients from CADASIL families can be identified via predictive genetic testing. In patients with advanced disease, the therapeutic impact will likely be more limited, as the accumulated brain damage cannot be reversed by therapies that prevent further $NOTCH3^{ECD}$ aggregation. However, a potential benefit on disease course cannot be excluded.

NOTCH3 cysteine correction

NOTCH3 cysteine correction is aimed at restoring the underlying genetic defect in CADASIL, namely restoring the uneven number of cysteine residues in one of the EGFr domains of NOTCH3.[5,6] Priority should be given to the targeting of exon 4, as the majority of severely affected CADASIL patients harbour mutations in this exon.[6] However, cysteine corrective exon skipping can in theory be applied to 18 of the 23 *NOTCH3* exons,[1] thus exon skipping strategies targeting exon 4 also serve as a proof-of-concept for skipping of other *NOTCH3* exons. Although *in vitro* analysis has shown that cysteine corrected NOTCH3 proteins retain normal signaling capacity, *in vitro* analysis of protein aggregation has been hampered by confounding effects of NOTCH3 overexpression in cell models, resulting in aspecific perinuclear accumulation (data not shown). Other approaches, such as scanning for intensely fluorescent targets (SIFT) in a single particle assay, assesses multimerization of small protein fragments, which may not reflect aggregation properties of the full length skip protein.[7,8] The use of *in vivo* models can circumvent some of the problems encountered in cell models and will, moreover, allow for direct measurement of NOTCH3 protein aggregation in the vessel wall.

To gain more insight into aggregation propensity of NOTCH3 cysteine corrected proteins, we studied a family with naturally occurring cysteine corrective exon skipping, in this case skipping of exon 9 (Chapter 3).[9] Family members with this *NOTCH3* variant had only minimal $NOTCH3^{ECD}$ aggregation, likely due to some mutant RNA products escaping splicing,

suggesting that cysteine corrected NOTCH3 proteins do not aggregate. The affected family members had a mild CADASIL phenotype, although an (additional) effect of the NOTCH3 mutation position cannot be ruled out.[10] Overall, the knowledge obtained from this unique family encourages the continued development of NOTCH3 cysteine correction as a treatment for CADASIL patients.

Inducing NOTCH3 cysteine correction in the Leiden humanized transgenic *NOTCH3*^{Arg182Cys} mouse model would allow for assessing the aggregation propensity of cysteine corrected proteins *in vivo*. The effect of treatment on protein aggregation could be assesses using the NOTCH3 score[11] and the GOM classification system (Chapter 2),[12] both reflecting (the consequences of) NOTCH3^{ECD} aggregation in the brain. The first exploratory *NOTCH3* exon skipping experiments were performed in the Leiden mouse model using the *NOTCH3* antisense oligonucleotides (ASOs) developed and tested *in vitro*.[1] Using both systemic and intraventricular administration of ASOs, this showed too low skipping levels in the brain vasculature to allow for reliable assessment of an effect on protein aggregation (data not shown). The low skipping levels are likely due to pharmacodynamics (i.e. insufficient delivery to the vascular smooth muscle cells) and also due to low skipping efficiencies of ASOs not optimized for *in vivo* experiments. For future studies, ASOs should be further developed and optimized in terms of target sequence, ASO chemistry and tissue-homing conjugates, in order to increase skipping efficacy and delivery to vascular smooth muscle cells (VSMCs). As the VSMCs are located within the vessel walls of the brain vasculature, ASO administration could be via the 'blood-side' of the blood-brain barrier (i.e. systemically), or via the 'brain-side' of the blood-brain-barrier (i.e. intracerebroventricularly or intrathecally).[13,14] The advantage of intrathecal administration is that the interval between ASO administration is probably longer compared to systemic administration, because ASOs have a long half-life in the central nervous system.[14,15] However, systemic administration is much less invasive and is easier to perform. Since limited literature is available on cerebral blood vessel wall delivery, the optimization of delivery of the ASOs to the VSMCs will be one of the most crucial challenges to overcome.

As an alternative to NOTCH3 cysteine correction on the mRNA level, NOTCH3 cysteine correction could be achieved by a permanent exon deletion on the DNA level.[16] We have shown this to be feasible *in vitro* using CRISPR/Cas9-based genome editing (Chapter 3).[9] The potential benefit of genome editing is that patients could receive a one-time-treatment and that effects may be permanent. Currently, several *ex vivo* CRISPR/Cas trials are on-going on cells obtained from patients, such as in hematopoietic stem and progenitor cells for hematologic disorders. The CRISPR/Cas-components, i.e. guide RNA and Cas9 or Cas12, can be delivered to the cells *ex vivo* using physical methods (e.g. electroporation), viral vectors (e.g. adeno-associated virus (AAV)-vectors) or biomaterials (e.g. nanoparticles) and then

re-administered to the patient.[17] In case of CADASIL and many other genetic diseases, the affected cells must be gene edited *in vivo*, thus viral vectors or biomaterials must be used as a delivery vehicle.[18] CRISPR/Cas components could, for example, be delivered systemically or intrathecally by AAV-vectors, provided that these vectors have high target specificity and low affinity with non-targeted tissue, which is currently not the case.[17,19] Once in the cell, gene editing efficiency also needs to be high enough, and genomic off-target effects should be negligible, as these genomic changes are also irreversible and might lead to adverse effects.[20]

The effect of permanent NOTCH3 cysteine correction using gene editing could be studied in three dimensional *in vitro* disease models that closely recapitulate human brain vessels. In collaboration with leading experts in the field of organ-on-a-chip models, our group is currently developing a CADASIL-vessel-on-a-chip model using reprogrammed induced pluripotent stem cells (iPSCs) from CADASIL patients. Previous studies by others showed that developing capillary vessels from CADASIL-derived iPSCs is feasible.[21] CADASIL-vessel-on-a-chip models will facilitate the assessment of the morphology and (dys) function of VSMCs, pericytes and endothelial cells in a controlled setting, and even in real time. Potentially, CADASIL-vessel-on-a-chip could also serve as an *in vitro* model to study the effect of ASO-based NOTCH3 exon skipping therapies, as well as to study $NOTCH3^{ECD}$ aggregation in the context of the vessel wall.

CADASIL endpoints and biomarkers in mice

The Leiden transgenic $NOTCH3^{Arg182Cys}$ mouse model is highly suitable for testing therapies targeting *NOTCH3* RNA and DNA as it contains the full length human *NOTCH3* gene, rather than only mouse or rat *Notch3*.[22–24] As mice develop $NOTCH3^{ECD}$ granules earlier than GOM deposits,[11] a therapeutic response can probably be observed earlier using the NOTCH3 score than using the GOM classification system. A pre-clinical study testing passive $NOTCH3^{ECD}$ immunotherapy showed no effect on $NOTCH3^{ECD}$ aggregation or GOM deposition in the transgenic rat genomic $Notch3^{R169C}$ mouse model, but did show a beneficial effect on cerebral hemodynamics.[2] These results illustrate that functional measures, such as cerebral hemodynamics, have additional value in pre-clinical studies. In contrast to other mouse models, the Leiden mouse model does not show cerebral blood flow deficits or ischemic brain damage (Chapter 2), possibly explained by a different genetic background or fundamentally different measurement techniques.[12,23,25] The Leiden mouse model does not show any signs of vessel wall thickening or mural cell degeneration as is seen in CADASIL patients, probably due to the relatively short life span of mice compared to patients (Chapter 2). Various studies testing hemodynamics have recently been performed in mouse models using a plethora of techniques, including ASL-MRI and laser Doppler flow analysis, often yielding contradictory results.[3,11,12,23–35] For example, cerebrovascular reactivity is

quantified using different measurement techniques between laboratories, as well as using different stimuli to induce increased blood flow, resulting in incomparable results between mouse models.[12,23,25,29]

CADASIL endpoints and biomarkers in patients

Validated outcome measures and biomarkers for CADASIL are a pre-requisite to be able to perform clinical trials. The benefit/risk-analysis performed by the regulator for therapy approval, requires knowledge of the risks (i.e. side-effects of treatment and disease burden if no therapy is given) and the clinical benefit upon treatment, which need to be assessed by an outcome measure that should quantify a therapeutic effect relevant to patients. These outcome measures can be hard clinical endpoints, such as the development of stroke, disability or death. Alternatively, endpoints can be measures for disease progression, for example cognitive performance measured on standardized cognitive scales (e.g. CAMCOG). Showing clinical benefit within the timeframe of a clinical trial (i.e. 1-2 years) is challenging for slowly progressive diseases like CADASIL, especially when therapies aim to slow down or halt disease progression. In order to reduce time span and sample size of a clinical trial, surrogate clinical endpoints can be used, as long as they are measures that anticipate future clinical benefit (e.g. brain atrophy or potentially fluid biomarkers).

Cognitive measures and disability could be used as surrogate endpoints, but require too large sample sizes in symptomatic patients.[36,37] In early disease stages such cognitive measures are likely not sensitive enough to be used as surrogate endpoints, even though some studies have reported executive dysfunction before the onset of stroke.[38] Possibly, developing an integrated measure for disease severity, such as the Unified Huntington's Disease Rating Scale used for Huntington's disease,[39] would allow for increased sensitivity to monitor changes in disease severity over short time intervals. Meanwhile, lacune load and brain atrophy on MRI may be feasible markers to detect differences in 1- or 2-year disease progression. Changes in WHM volume can be measured sensitively over time, including in pre-symptomatic patients, but WMH volume is probably not the best surrogate marker, because it does not correlate with clinical disease severity or progression (Chapter 4).[40–42] Diffusion tensor imaging (DTI) sensitively measures brain damage, was previously estimated to require less patients in a clinical trial than other neuroimaging or cognitive measures, and might be the most promising neuroimaging markers for pre-symptomatic patients.[37,43] Possibly, neuroimaging measures of specific brain regions are suitable surrogate markers.[44,45] Complementary to neuroimaging markers, serum NfL levels could be used as blood biomarker for symptomatic patients, as they correlate with (ischemic) neuronal damage, and cognitive impairment in CADASIL (Chapter 5).[46,47] In a long-term follow-up study, however, there were no significant differences in 18-year increase in serum NfL in pre-symptomatic or mildly affected patients compared to controls, indicating that

NfL is mainly a biomarker for symptomatic patients. More research on the temporal profile of serum NfL is needed, in order to determine the relative contribution of acute ischemia and overall disease progression to serum NfL levels in CADASIL patients. This will help determining whether serum NfL could serve as a biomarker responsive to a therapy, as is the case for Multiple Sclerosis.[48,49]

CADASIL vessel wall pathology seems to precede neuronal damage, therefore markers arising due to blood vessel wall damage might be more sensitive to early disease stages than neuroimaging markers. There have been reports of seemingly contradictory results on resting cerebral blood flow (CBF) and cerebrovascular reactivity (CVR) in patients, probably caused by the heterogeneity of techniques and patients samples used.[50–56] Overall, it seems reduced CBF and CVR can only be reliably measured in severely affected patients, using state-of-the-art analyses, such as CVR measurements of selected brain regions and measurements of temporal dynamics of the vascular reactivity.[54–56]

The amount of $NOTCH3^{ECD}$ aggregates or classification of GOM deposits visualized in blood vessel walls in skin biopsies could be associated with disease severity or progression, and could serve as a prognostic or monitoring biomarker. However, a limitation of these markers is that $NOTCH3^{ECD}$ aggregates and GOM deposits in skin biopsies might not reflect $NOTCH3^{ECD}$ aggregation and GOM deposits in the cerebrovasculature, and that these markers would imply obtaining repeated skin biopsies.

The retina and retinal vessels might reflect changes in the brain and brain vessels, as the retina has the same embryonal origin as the brain and is also affected by CADASIL pathology. In humans, the retina can be visualized non-invasively using optimal coherence tomography (OCT), thereby providing a high resolution three dimensional image of the retina. Using OCT, the thickness of retinal arteries was found to be increased in CADASIL patients,[57–59] and the thickness of the retinal nerve fibre layer was reported to be reduced.[60,61] Retinal biomarkers are attractive as they are non-invasive and cheaper than neuroimaging biomarkers, but further investigations are needed, as currently available data is based on only a few studies with limited sample sizes.

The potential blood biomarkers Endostatin, HTRA1, IGF-BP1 and TGF-ß were not associated with disease severity (Chapter 4). Biomarkers in cerebrospinal fluid (CSF) of CADASIL patients has not been studied extensively yet and may be worth further investigation.[62–64] Identification of fluid biomarkers in blood or CSF would likely be most promising using sensitive multiplex immunoassays or mass spectrometry approaches. Quantification of such identified biomarkers, should preferably be performed using sensitive techniques such as ultra-sensitive single molecule assays.

CADASIL disease severity and progression

Variability in CADASIL disease course has long been recognized, even between patients of the same family, and in monozygotic twins.[65,66] This variability was also observed in the 18-year follow-up study we performed, which also showed that some patients can remain remarkably stable over the course of almost two decades (Chapter 4). Disease modifier known so far are classical vascular risk factors, such as smoking and high blood pressure,[65,67,68] and the *NOTCH3*^{cys} mutation position.[10] However, these modifiers do not explain all of the disease variability. Additional disease modifiers must be present and could be either genetic or environmental,[67,69,70] including variants in proteins known to be associated with the molecular disease pathology, such as HTRA1, LTBP-1, TIMP3 or proteins of the ADAM17/HB-EGF/(ErbB1/ErbB4) pathway.[71–73]

Future perspectives

Within 25 years after the discovery of *NOTCH3* as the causative gene for CADASIL, the CADASIL research field has significantly progressed and has acquired much knowledge on disease pathomechanisms and disease course. Several CADASIL mouse models have been developed, many pre-clinical and clinical measures for disease severity have been described, and the proof-of-concept of several therapeutic approaches has been reported. Now, the time is ripe for the CADASIL research field to advance pre-clinical therapy development and aim for clinical trial readiness. This involves obtaining pre-clinical data showing therapy efficacy and safety, as well as developing validated (surrogate) endpoints for future clinical trials. In order to validate surrogate endpoints, a short-term natural history study must be performed to select the best outcome measures.

Key messages from this thesis

- Granular osmiophilic material (GOM) deposits evolve over time with respect to size, morphology and number, which can be classified in a five-stage GOM deposit classification system (Chapter 2).

- The Leiden humanized transgenic *NOTCH3*^{Arg182Cys} mouse model recapitulates early signs of CADASIL disease pathology (i.e. NOTCH3^{ECD} aggregation and GOM deposition), but not other signs of disease pathology observed in CADASIL patients (i.e. blood vessel wall thickening, brain parenchyma pathology and cognitive dysfunction) (Chapter 2).

- Natural occurring *NOTCH3* exon skipping is associated with reduced NOTCH3 protein aggregation in CADASIL patients, suggesting that cysteine corrected NOTCH3 proteins do not aggregate (Chapter 3).

- Long-term disease progression is highly variable in CADASIL patients, and a subset of patients remains remarkably stable over the course of 18 years (Chapter 4).

- Cerebral blood flow and cerebrovascular reactivity (measured by gradient-echo phase-contrast MRI) and blood levels of the proteins HTRA1, Endostatin, IGF-BP1 and TGF-ß (measured by ELISA) are not suitable as biomarkers for CADASIL (Chapter 4).

- Serum Neurofilament Light-chain (NfL) is a blood biomarker for CADASIL in symptomatic patients, reflecting lacune load and brain atrophy, and correlates with disease severity, disease progression and survival (Chapter 5).

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