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DISCUSSION AND FUTURE PERSPECTIVES

The aim of this thesis was to work towards pre-clinical proof-of-concept for NOTCH3 cysteine corrective exon skipping as a rational therapeutic approach for CADASIL. To address all aspects required for therapeutic development, the work performed for this thesis included not only *in vitro* testing of *NOTCH3* exon skipping in CADASIL patient derived vascular smooth muscle cells and studies into the function of the cysteine corrected proteins, but also the generation of a relevant humanized *in vivo* model, pre-clinical biomarker development, and studies defining prevalence, spectrum and characteristics of *NOTCH3* mutations worldwide.

There is a high unmet medical need in CADASIL. Currently, only palliative care and symptomatic treatment can be offered,[1] and there are no effective therapies that can delay the onset or progression of physical and cognitive dysfunction. The window of opportunity for treatment probably spans mainly the pre-symptomatic and early symptomatic stages of the disease, when no or only minimal brain white matter hyperintensities are present, and presumably no or only little irreversible damage has occurred in the vessel wall or to the brain tissue. The fact that asymptomatic family members of a CADASIL patient can have predictive molecular genetic testing, in young adults sometimes decades prior to the onset of clinical symptoms, in principle permits therapeutic intervention before irreversible damage has been done. The therapeutic approach described in this thesis is an antisense oligonucleotide- based strategy. However, many of the steps needed to bring this approach to clinical trial readiness also apply to other potential therapeutic approaches. Developing a more comprehensive framework for CADASIL therapeutic development is thus of wider benefit.

Cysteine correction of NOTCH3 is a therapeutic approach which directly targets the underlying defect in CADASIL: and uneven number of cysteines within EGFr. We have found that correcting the uneven number of cysteines using antisense-mediated exon skipping is feasible *in vitro* and does not abrogate normal protein processing and functionality. The crucial question that remains to be addressed is whether the modified NOTCH3 proteins formed after cysteine corrective exon skipping indeed have a reduced multimerization tendency and reduced aggregation properties. To address this question, we have generated stable cell lines with an inducible NOTCH3 skip protein expression. These cell lines can be used to measure NOTCH3 protein solubility and degradation as a read-out for protein aggregation.[2-4] An important limitation of such cell models, however, is protein overexpression. NOTCH3 overexpression is inherently associated with intracellular NOTCH3 protein aggregation, which may confound a reliable assessment of CADASIL- associated NOTCH3 protein aggregation. We are currently investigating an alternative *in vitro* model mimicking cysteine correction in (primary) cells with endogenous

NOTCH3 expression, using CRISPR-Cas9 genome editing technology.[5] Although potentially a more representative model, this approach is challenging as primary cells have a limited life-span and genome editing is not always efficient. Finally, we are collaborating with the LUMC mass-spectrometry group to apply advanced mass-spectrometry techniques in order to assess disulphide bridge formation in cysteine corrected NOTCH3 protein fragments.[6]

Proof of concept for the hypothesis that NOTCH3 cysteine correction reduces vascular toxic NOTCH3 aggregation will have to come from *in vivo* studies. Studies towards this *in vivo* proof of concept are now enabled by the human *NOTCH3* transgenic mouse model we generated (Chapter 5). The NOTCH3 score which we developed in this model can be used to determine the effect of a therapeutic intervention on NOTCH3 accumulation, as a marker for therapeutic efficacy. More detailed characterization of the *NOTCH3* transgenic mice is ongoing, including assessment of vascular smooth muscle cell degeneration and cerebrovascular reactivity. So far, the human *NOTCH3* transgenic mice have not shown any obvious signs of stroke or cognitive impairment. Studies formally assessing cognition and motor function are on-going, but we expect that, analogous to most other CADASIL mouse models, these mice will not develop measurable motor deficits or cognitive dysfunction. A different genetic background or additional aggravating factors, such as hypertension or nicotine exposure,[7, 8] may be needed to provoke a clinical phenotype in the mice. The role of hypotension in CADASIL is not well studied, but could theoretically provoke the occurrence of lacunar infarcts in watershed areas of the brain. However, the short life span and the anatomy of the mouse brain may be the most important limiting factors in recapitulating the full CADASIL phenotypic spectrum in mice. This is an important limitation, as stroke or cognition can therefore not be used as therapeutic read-outs. Given this limitation and considering the window of opportunity for pre-symptomatic intervention in CADASIL, it may be more important to focus on translational markers for disease progression in the pre- and early symptomatic stages of the disease. If these markers prove to correlate with disease severity and progression in humans, they can then be used as pre-clinical and clinical surrogate markers. The NOTCH3 score we developed in the mice is such a potential translatable biomarker, as it progresses as the mouse ages and this same NOTCH3 accumulation can also be detected in skin arterioles of CADASIL patients, decades before the onset of clinical symptoms.[9, 10] We are currently investigating whether the NOTCH3 score can be reliably measured in CADASIL skin biopsies and whether it correlates with disease severity and progression.

Although cysteine corrective exon skipping is an elegant strategy to bypass the pathogenic unpaired cysteine, there are some important limitations. Firstly,

it still remains to be determined whether this approach indeed reduces NOTCH3 aggregation *in vivo*. Also, the cysteine corrective exon skips we optimized require a combination of AONs for effective exon skipping and skipping efficiencies are not always high enough to ensure therapeutic efficacy. An alternative treatment strategy, which we are testing in parallel, is NOTCH3 downregulation. Our study on individuals with a *NOTCH3* stop mutation (Chapter 3) provides a solid rationale for this approach and has the added advantage that a single effective RNAse H inducing AON could be used for all CADASIL patients, irrespective of their mutation. The underlying concept is that a reduced expression of total NOTCH3 will also lower the amount of mutant NOTCH3, thereby theoretically decreasing NOTCH3 protein aggregation. However, a potential pitfall of this approach is that adult exposure to pharmacologically induced reduction of NOTCH3 is not comparable to a congenitally reduced NOTCH3 expression due to a stop mutation, with potential compensatory mechanisms. Moreover, a complete absence of NOTCH3, due to a homozygous stop mutation, does cause a severe cerebrovascular phenotype in humans,[11] and it is not well known how much NOTCH3 is required for normal vessel wall function in adults. Finally, it should be noted that the exact same stop mutation we identified in healthy individuals, was later found in a family with cerebral small vessel disease.[12] Therefore, although most studies indicate that there is some flexibility in the amount of NOTCH3 needed for normal functioning, the exact effects of loss of NOTCH3 function, both in the context of CADASIL and in the context of blood vessel development and maintenance, requires further study.

The major hurdle faced in obtaining *in vivo* proof of concept for NOTCH3 cysteine correction or NOTCH3 downregulation, is to attain sufficient levels of exon skipping or downregulation in the target cells, namely the vascular smooth muscle cells in the small-to medium sized arteries of the brain. Experiments are on-going in our lab to determine which route is most optimal for *NOTCH3* AON delivery into the cerebrovasculature, via the systemic circulation, or via the cerebrospinal fluid. Systemic administration of AONs, through intravenous, intraperitoneal of subcutaneous injection, is technically most straightforward. However, systemic administration causes the vast majority of AONs to be taken up by the liver and kidneys.[13] While the blood brain barrier precludes most AON chemistries from entering the brain, the cerebral vascular smooth muscle cells should in principle be accessible, but little is known about vessel wall delivery.[14] Administration to the cerebrospinal fluid, via intraventricular injection in mice or intrathecal injection in humans, is a more invasive administration route, but has the advantage that high concentrations of AONs in cerebrospinal fluid are easily achieved and half-life of AONs in cerebrospinal fluid is longer than in serum.[15] Next to delivery route, other ways to increase AON efficacy are improved AON chemistries, which are continually

being developed. For example, AONs with a 2'-O-methoxy-ethyl chemistry may be better tolerated in the cerebrospinal fluid than 2OMePS AONs, and a recent study demonstrated uptake of tricyclo AONs in the brain after systemic administration.[16]

Another challenge in CADASIL therapeutic development is the lack of feasible clinical read-outs. Clinical read-out development is hampered by a pronounced clinical variability, likely attributable to genetic and environmental modifiers. Despite studies in large groups of patients, feasible clinical read-outs or biomarkers have not yet been identified. Our finding of an unexpected high population frequency of *NOTCH3* mutations (Chapter 6) may have far-reaching implications for our understanding of CADASIL disease variability, as it shows that we have currently only identified a small subset of (severe) CADASIL patients. Probably most individuals with a *NOTCH3* mutation who have not yet been diagnosed, have a milder disease course than the classical mid-adult onset CADASIL. Such a milder disease course has been described[18, 19] and is increasingly encountered in our CADASIL outpatient clinic. A strong genotype-phenotype correlation would enable classification of *NOTCH3* mutations into predisposing to severe disease or predisposing to mild disease. In this way we might be able to specifically enrol only patients predisposed to severe disease into biomarker studies, which should be facilitated by the more narrow severe disease spectrum and reduced disease variability.

In the not so distant future, it is likely that every individual will have his or her exome or genome sequenced. For *NOTCH3* specifically, routine exome or genome sequencing will expose the large number of individuals with a cysteine altering *NOTCH3* mutation who now remain undiagnosed. Identification of these individuals will be important in our further understanding of the phenotypic spectrum associated with *NOTCH3* mutations and the identification of other genetic and environmental factors which mitigate disease progression, some of which may lead to strategic interventions.

KEY MESSAGES OF THIS THESIS

- Only cysteine altering *NOTCH3* mutations are indisputably causative of CADASIL, other variants should be considered polymorphisms until proven otherwise. Classification of non-cysteine altering *NOTCH3* mutations can only be attained in the context of brain MRI, clinical and family history and skin biopsy including NOTCH3 immunohistochemistry and electron microscopy analysis. (Chapter 2)
- *NOTCH3* loss of function mutations do not cause CADASIL and there is flexibility in the amount of NOTCH3 needed for normal health and development. (Chapter 3)
- Cysteine correction of NOTCH3 can be achieved via antisense- mediated exon skipping in CADASIL patient derived VSMCs. A cysteine corrected NOTCH3 protein lacks the corruptive mutated EGFr domain, whilst retaining functionality. (Chapter 4)
- Human genomic *NOTCH3* transgenic mice recapitulate the CADASIL vascular phenotype, and are a good model for studying therapies that target human NOTCH3 at the genomic, (pre-)mRNA or protein level. (Chapter 5)
- The NOTCH3 score is a quantitative measure for progressive NOTCH3 accumulation in mice, and can potentially be translated to humans. (Chapter 5)
- EGFr cysteine altering *NOTCH3* mutations have a worldwide frequency of 3.4/1000. The phenotypic spectrum associated with these *NOTCH3* mutations is probably much broader and milder than currently recognized. (Chapter 6)
- Mutations in EGFr domains 1-6 predispose to a more severe disease course, whilst mutations outside of these domains predispose to a milder disease course. This genotype-phenotype correlation may partially explain CADASIL disease variability. (Chapter 6)

REFERENCES

1. del Rio-Espinola A, Mendioroz M, Domingues-Montanari S, Pozo-Rosich P, Sole E, Fernandez-Morales J, Fernandez-Cadenas I, Montaner J. CADASIL management or what to do when there is little one can do. *Expert Rev Neurother* 2009 February;9(2):197-210.
2. Monet-Lepretre M, Haddad I, Baron-Menguy C, Fouillot-Panchal M, Riani M, Domenga-Denier V, Dussaule C, Cognat E, Vinh J, Joutel A. Abnormal recruitment of extracellular matrix proteins by excess Notch3ECD: a new pathomechanism in CADASIL. *Brain* 2013 June;136(Pt 6):1830-45.
3. Takahashi K, Adachi K, Yoshizaki K, Kunimoto S, Kalaria RN, Watanabe A. Mutations in NOTCH3 cause the formation and retention of aggregates in the endoplasmic reticulum, leading to impaired cell proliferation. *Hum Mol Genet* 2010 January 1;19(1):79-89.
4. Meng H, Zhang X, Yu G, Lee SJ, Chen YE, Prudovsky I, Wang MM. Biochemical characterization and cellular effects of CADASIL mutants of NOTCH3. *PLoS One* 2012;7(9):e44964.
5. Young CS, Hicks MR, Ermolova NV, Nakano H, Jan M, Younesi S, Karumbayaram S, Kumagai-Cresse C, Wang D, Zack JA, Kohn DB, Nakano A, Nelson SF, Miceli MC, Spencer MJ, Pyle AD. A Single CRISPR-Cas9 Deletion Strategy that Targets the Majority of DMD Patients Restores Dystrophin Function in hiPSC-Derived Muscle Cells. *Cell Stem Cell* 2016 February 10.
6. Switzar L, Nicolardi S, Rutten JW, Oberstein SA, Aartsma-Rus A, van der Burgt YE. In-Depth Characterization of Protein Disulfide Bonds by Online Liquid Chromatography-Electrochemistry-Mass Spectrometry. *J Am Soc Mass Spectrom* 2016 January;27(1):50-8.
7. Adib-Samii P, Brice G, Martin RJ, Markus HS. Clinical spectrum of CADASIL and the effect of cardiovascular risk factors on phenotype: study in 200 consecutively recruited individuals. *Stroke* 2010 April;41(4):630-4.
8. Chabriat H, Herve D, Duering M, Godin O, Jouvent E, Opherk C, Alili N, Reyes S, Jabouley A, Zieren N, Guichard JP, Pachai C, Vicaut E, Dichgans M. Predictors of Clinical Worsening in Cerebral Autosomal Dominant Arteriopathy With Subcortical Infarcts and Leukoencephalopathy: Prospective Cohort Study. *Stroke* 2016 January;47(1):4-11.
9. Lesnik Oberstein SA, van Duinen SG, van den Boom R, Maat-Schieman ML, van Buchem MA, van Houwelingen HC, Hegeman-Kleinn IM, Ferrari MD, Breuning MH, Haan J. Evaluation of diagnostic NOTCH3 immunostaining in CADASIL. *Acta Neuropathol* 2003 August;106(2):107-11.
10. Joutel A, Favrole P, Labauge P, Chabriat H, Lescoat C, Andreux F, Domenga V, Cecillon M, Vahedi K, Ducros A, Cave-Riant F, Bousser MG, Tournier-Lasserve E. Skin biopsy immunostaining with a Notch3 monoclonal antibody for CADASIL diagnosis. *Lancet* 2001 December 15;358(9298):2049-51.
11. Pippucci T, Maresca A, Magini P, Cenacchi G, Donadio V, Palombo F, Papa V, Incensi A, Gasparre G, Valentino ML, Preziuso C, Pisano A, Ragno M, Liguori R, Giordano C, Tonon C, Lodi R, Parmeggiani A, Carelli V, Seri M. Homozygous NOTCH3 null mutation and impaired NOTCH3 signaling in recessive early-onset arteriopathy and cavitating leukoencephalopathy. *EMBO Mol Med* 2015 June;7(6):848-58.
12. Moccia M, Mosca L, Erro R, Cervasio M, Allocca R, Vitale C, Leonardi A, Caranci F, Del Basso-De Caro ML, Barone P, Penco S. Hypomorphic NOTCH3 mutation in an Italian family with CADASIL features. *Neurobiol Aging* 2015 January;36(1):547.
13. Verhaart IE, Tanganyika-de Winter CL, Karnaoukh TG, Kolfschoten IG, de Kimpe SJ, van Deutekom JC, Aartsma-Rus A. Dose-dependent pharmacokinetic profiles of 2'-O-methyl phosphorothioate antisense oligonucleotidesin mdx mice. *Nucleic Acid Ther* 2013 June;23(3):228-37.
14. Boursereau R, Donadieu A, Dabertrand F, Dubayle D, Morel JL. Blood brain barrier precludes the cerebral arteries to intravenously-injected antisense oligonucleotide. *Eur J Pharmacol* 2015 January 15;747:141-9.

15. Evers MM, Toonen LJ, van Roon-Mom WM. Antisense oligonucleotides in therapy for neurodegenerative disorders. *Adv Drug Deliv Rev* 2015 March 20.
16. Goyenvalle A, Griffith G, Babbs A, Andaloussi SE, Ezzat K, Avril A, Dugovic B, Chaussenot R, Ferry A, Voit T, Amthor H, Buhr C, Schurch S, Wood MJ, Davies KE, Vaillend C, Leumann C, Garcia L. Functional correction in mouse models of muscular dystrophy using exon-skipping tricyclo-DNA oligomers. *Nat Med* 2015 March;21(3):270-5.
17. Rutten J, van DS, Lesnik OS. Letter by Rutten et al Regarding Article, "Cysteine-Sparing CADASIL Mutations in NOTCH3 Show Proaggregatory Properties In Vitro". *Stroke* 2015 June;46(6):e153.
18. Moreton FC, Razvi SS, Davidson R, Muir KW. Changing clinical patterns and increasing prevalence in CADASIL. *Acta Neurol Scand* 2014 September;130(3):197-203.
19. Lee YC, Yang AH, Soong BW. The remarkably variable expressivity of CADASIL: report of a minimally symptomatic man at an advanced age. *J Neurol* 2009 June;256(6):1026-7.