Unraveling The Broad NOTCH3-associated Small Vessel Disease Spectrum: from CADASIL to nonpenetrance

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Chapter 7

Discussion & Future Perspectives

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Until 2016, *NOTCH3*^{cys} variants were believed to be rare and fully penetrant, invariably resulting in a classical severe CADASIL phenotype characterized by mid-adult onset of strokes, progressive vascular cognitive impairment and disability. However, *NOTCH3*^{cys} variants are not rare at all, occurring at a surprisingly high frequency of approximately 1:300 in the general population. This thesis demonstrates that these *NOTCH3*^{cys} variants in the general population are associated with a manifold milder SVD phenotype than what is typically seen in CADASIL patients with nonpenetrance even occurring up to the 8th decade. These mild phenotypes are not only found in the general population, but can also sometimes be observed in known CADASIL pedigrees, where family members with the familial *NOTCH3*^{cys} variant can remain asymptomatic with completely normal brain MRI up to the 6th decade, while their first-degree relatives may exhibit a classical severe CADASIL phenotype.

The position of the *NOTCH3*^{cys} variant within the EGFr domains is by far the most important modifier of NOTCH3-SVD severity. In this thesis, I have enhanced the NOTCH3-SVD genotype-based risk stratification, by stratifying *NOTCH3*^{cys} variants into three risk categories. *NOTCH3*^{cys} variants in the high-risk EGFr (HR-EGFr) domains are associated with the most severe NOTCH3-SVD phenotype, which is the phenotype most physicians would recognize as CADASIL. Conversely, *NOTCH3*^{cys} variants in low-risk EGFr (LR-EGFr) domains are predominantly identified in community-dwelling individuals and are associated with the mildest end of the NOTCH3-SVD spectrum. These individuals can even be asymptomatic in their 8th decade with only a very low burden of SVD neuroimaging markers on brain MRI. *NOTCH3*^{cys} variants in the medium-risk EGFr (MR-EGFr) domains are relatively common in both CADASIL pedigrees and community-dwelling individuals, and it follows that these *NOTCH3*^{cys} variants are associated with the most variable NOTCH3-SVD phenotype, ranging from classical CADASIL to very mild subclinical phenotypes. In addition to *NOTCH3*^{cys} variant position, the most important disease modifiers are sex, hypertension and diabetes.[1-7]

In the future, further refinement of the NOTCH3-SVD risk classification can be achieved by incorporating data from additional CADASIL cohorts and whole-exome sequencing databases encompassing diverse populations worldwide. This is important as previous studies have highlighted that there are notable phenotypic differences among various

ethnic backgrounds. For instance, it has been shown that Asian CADASIL patients, for yet unknown reasons, have a much higher occurrence of intracerebral hemorrhage than CADASIL patients from Western populations.[8]

Accurate individualized NOTCH3-SVD disease prediction is important not only for CADASIL patients, but also for individuals in whom a *NOTCH3*^{cys} variant is found as an incidental finding by whole-exome sequencing for unrelated disorders. The latter group will only expand in the coming years as whole-exome sequencing is becoming faster, more cost-effective and more widely utilized in clinical practice. Nevertheless, even when accounting for the aforementioned disease modifiers, a substantial proportion of NOTCH3-SVD variability remains unexplained, illustrated by the fact that NOTCH3-SVD severity can still be highly variable between individuals with the same *NOTCH3*^{cys} variant and a similar cardiovascular risk burden, even within the same family. This indicates there still must be additional important genetic and environmental factors involved, which have not yet been identified.

The substantial technological advancements in genomics, transcriptomics, proteomics, and the availability of large population-based whole-exome sequencing biobanks, such as the UK Biobank, provide immense potential for identifying many of these unknown genetic and environmental modifiers in the near future. Many of these genetic modifiers are expected to be identified in genes that encode for, interact with or regulate the expression of proteins involved in maintaining the extracellular matrix homeostasis in the small arteries of the brain, which have been shown to be implicated in not only the pathogenesis of CADASIL, but also in sporadic SVD.[9] This hypothesis is supported by recent studies demonstrating that a higher burden of SVD was associated with relatively common variants in the *HTRA1* gene, of which the protein has been shown to co-aggregate with mutant NOTCH3 proteins in the extracellular matrix of small cerebral arteries in CADASIL patients.[10] Interestingly, *HTRA1* is also the causative gene of another autosomal dominant SVD (HTRA1 autosomal dominant SVD) as well as the rare autosomal recessive SVD "Cerebral Autosomal Recessive Arteriopathy with Subcortical Infarcts and Leukoencephalopathy" (CARASIL), which both have striking similarities with CADASIL.[11,12] The integration of various genetic modifiers into a polygenic risk score for NOTCH3-SVD will undoubtedly represent the next step in advancing individualized disease prediction, which will enable the identification of all individuals who are likely to benefit from preventive measures and therapeutic treatment.

The studies described in this thesis provide evidence supporting abnormal NOTCH3 aggregation as the primary pathophysiological mechanism in NOTCH3-SVD, as EGFr risk category was found to be strongly associated with vascular NOTCH3 aggregation load, whereas no association was found between EGFr risk category and NOTCH3 signalling properties. As HR-EGFr patients have been shown to have a higher load of GOMs in skin- and brain vasculature,[13] our hypothesis is that HR-EGFr variants are more prone to co-aggregate with other extracellular matrix proteins.[10,14,15] Although 3D protein modelling with Alphafold revealed some differences in spatial localization between EGFr risk categories, these differences were insufficient to provide a convincing explanation for the disparity in aggregation propensity between EGFr risk categories. Alternatively, the differences in NOTCH3 aggregation propensity might be attributed, at least in part, to the recently identified non-enzymatic cleavage sites at Asp–Pro bonds, which are situated between HR-EGFr 1–2, HR-EGFr 2–3, and in close proximity to HR-EGFr domain 26.[16,17] Additional *in vitro* and *in vivo* studies are necessary to fully elucidate the mechanism underlying the differences in NOTCH3-SVD severity between EGFr risk categories.

A modifying role for aberrant NOTCH3 signalling on NOTCH3-SVD severity is, however, not completely ruled out. From a pathophysiological perspective, it is plausible that aberrant NOTCH3 signalling may increase the susceptibility to SVD, considering the established importance of NOTCH3 signalling for the vascular integrity of small cerebral arteries.[18] This notion is supported by several reports demonstrating that heterozygous *NOTCH3* loss of function variants are associated with an increased risk of SVD,[19-21] although the penetrance is highly variable. Previous studies comparing the signalling properties of wildtype and mutant NOTCH3 proteins have yielded conflicting results. Some studies reported lower signalling properties of mutant NOTCH3,[22,23] while others found no differences between wildtype and mutant,[24,25] or even higher signalling properties of mutant NOTCH3.[26] Determining the precise impact of specific *NOTCH3*^{cys} variants on NOTCH3 signalling, as well as whether these alterations in signalling also exert a disease modifying effect on NOTCH3-SVD severity, remains a relevant objective for future research.

Considering the high frequency of *NOTCH3*^{cys} variants in the population, numerous individuals worldwide are at risk for developing NOTCH3-SVD with ischemic strokes and vascular cognitive impairment, making *NOTCH3*^{cys} variants a significant contributor to the global burden of dementia. Based on the current world population of 8 billion, 60

million individuals in the world are estimated to harbor a *NOTCH3*^{cys} variant, emphasizing the potential impact preventive measures and therapeutic treatment tailored to NOTCH3-SVD could have on global health. Unfortunately, there is currently no curative therapy for CADASIL, but active NOTCH3 immunization has recently been shown to be a promising therapeutic approach, as it resulted in a substantial reduction of capillary NOTCH3 aggregates in a CADASIL mouse model after a 4 month treatment regimen.[27] Given the substantial influence of cardiovascular risk factors on NOTCH3-SVD progression, cardiovascular risk management in CADASIL patients is warranted. Antiplatelet therapy is commonly employed in the management of CADASIL to mitigate the risk of ischemic stroke, as it has shown efficacy in reducing ischemic stroke risk in sporadic SVD following lacunar stroke.[28] However, it is important to note that due to a lack of studies there is currently no evidence supporting the efficacy of antiplatelet therapy specifically for CADASIL, highlighting the need for further research in this area.

In conclusion, the NOTCH3-SVD spectrum is much broader than previously thought, ranging from a classical severe CADASIL phenotype to non-penetrance up to the 8th decade in the general population. The most important disease modifier is *NOTCH3*^{cys} variant position, followed by sex and cardiovascular risk factors. Through the development of our novel 3-tiered *NOTCH3* EGFr risk classification, the accuracy of individualized NOTCH3-SVD disease prediction for both CADASIL patients and individuals with incidentally identified *NOTCH3*^{cys} variants has been significantly improved. Finally, this thesis provides evidence supporting NOTCH3 aggregation as the primary pathophysiological mechanism in NOTCH3-SVD.

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