# Molecular pathology of hereditary cerebral hemorrhage with amyloidosis-Dutch type

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# Chapter 7

# Discussion

## Aim of the study & central findings

The general aim of this thesis was to decipher the molecular pathogenesis of HCHWA-D. Since no proven therapeutic treatment exists to prevent or even delay the disease onset, the understanding of underlying pathomechanisms in HCHWA-D is important. It may help discovering new therapeutic targets and biomarkers that can be used to assess the efficacy of candidate drugs in treatment trials.

The main finding of the work presented in this thesis is that TGFβ deregulation plays a central role in HCHWA-D pathology. This deregulation is demonstrated using HCHWA-D brain material. At the gene expression level, on individual genes, TGFβ1, its receptors and TGFβ-induced genes are upregulated (chapter 3). At the pathway level, in the transcriptomic study, extracellular matrix (ECM)-pathways related to TGFβ are increased (chapter 6). Lastly at the protein level using histology, we showed the actual activation of the TGFβ signaling cascade. First by showing the accumulation of its activated transcription factor (pSMAD2/3; chapter 3) in some angiopathic vessels, then by identifying the presence of the pro-fibrotic collagen 1 (col1) protein, a major TGFβ-induced genes, in amyloid-laden vessels (chapter 5). By selecting patients at different disease stages, we discovered that pSMAD2/3 and col1 accumulation in angiopathic vessel walls are correlated with CAA load (chapter 3 & 5).

We show that TGFβ deregulation correlates with disease severity. However, disease severity in HCHWA-D is also characterized by the higher occurrence of secondary remodelling of angiopathic vessels (1). In an initial study, we identified vascular calcifications, a specific secondary vascular remodelling, to be the cause of a recently described neuroimaging feature of advanced disease stage in HCHWA-D (2). In the subsequent study, we assessed the presence of osteopontin (OPN) in these calcifications, as described in other non-Aβ related vasculopathies in link with TGFβ increase (3). We further discovered a topographical association of pSMAD2/3 and OPN in these calcifications and we also linked their gradual accumulation with the calcification process (chapter 5).





## Study of pathomechanisms in human brain tissue material

### Advantages & Difficulties

The use of human brain material is undeniably a unique chance to study pathomechanisms in relevant tissue and has a high scientific value. Using patient tissue gives access to the whole complexity of the disease in comparison to pathological processes *in vitro* which lack the cellular and physiological context or *in vivo* studies in animal model which cannot fully recapitulate human brain diseases.

Despite these advantages, patient brain material is underused in research mostly because its utilization remains challenging (4). The principal drawback is the heterogeneity of samples due to individual differences (age, gender, health, body mass, life style), prior medication exposure and additional co-morbidities, which are confounding factors. Secondly, for gene expression analysis, the RNA quality is variable, mostly due to the circumstances of death (agonal stress, (5)) but other factors such as postmortem interval, dissection protocols and storage conditions can influence the RNA integrity as well. Because the RNA quality is generally lower than for RNA isolated from cell cultures or animal models, it requires testing and optimisation of standard procedures, as discussed in chapter 6. Thirdly, the material is scarce, which might be restrictive in experimental design. Lastly, postmortem human tissue reflects the end-stage of the disease and therefore cannot replace cellular and animal research models where early events in illness can be studied.

### Frontal *versus* occipital pathology in HCHWA-D

Previous CAA neuropathological studies suggested that CAA pathology was more prominent in the occipital lobes than in the frontal lobes (chapter 1). We studied these two areas as they were presumed to represent different disease stages. Some histopathological findings (other than β-amyloid vascular accumulation) were indeed more prominent occipitally. We discovered both perivascular ring of pSMAD2/3 granules (chapter 3) and cortical calcifications of angiopathic vessels (which are a very advanced disease stage) predominantly occipitally (chapter 4), although a direct link between these two findings remain to be demonstrated. However, at the transcriptomic level, we did not find evidence that frontal and occipital cortexes of HCHWA-D were differently affected (chapter 6). Also the amount of angiopathic vessels showed no significant difference between the frontal and the occipital cortex (chapter 3). Nevertheless, one finding of this thesis is that the study of patients with different disease stages is more informative of pathomechanisms than the study of different brain areas.

The correlation of the different CAA load with pSMAD2/3 accumulation independently of the brain area, revealed TGFβ-related pathomechanisms (chapter 3).

- Pathological disease severity in HCHWA-D is comprised of CAA load plus additional secondary vascular remodeling
- Gene expression in frontal and occipital cortex is similar but secondary vessel pathology showed more perivascular changes and vascular calcifications occipitally

## Consequences of TGFβ activation

TGFβs are pleiotropic cytokines involved in a variety of biological effects, modulating ECM metabolism, cell cycle as well as immune response (6). Transgenic mice overexpressing TGFβ1 in brain cells have been extensively studied to understand the influence of this cytokine on the vasculature, on neuroinflammation and on Aβ deposition (7,8). Results of this thesis, in light of knowledge from previous neuropathological and transgenic mice studies, further implicate TGFβ in HCHWA-D phenotypic variability.

### Effects of TGFβ on the cerebrovasculature

#### *Cerebrovascular fibrosis*

The pathogenesis of cerebrovascular fibrosis has been studied in mouse model overexpressing TGFβ1 in astrocytes (GFAP-TGFβ1, (9)). It starts by a perivascular astrogliosis inducing an early thickening of vascular basement membranes (3-4 months) by ECM proteins, followed by the deposition of amyloid fibrils by 6 months of age. When these mice are crossed with mice overexpressing human APP (hAPP) in neurons (GFAP-TGFβ1/PDGF-hAPP), amyloid vascular deposits are detected even earlier (2-3 months). Remarkably, another mouse model with a neuron-specific TGFβ1 inducible overexpression, develop a similar angiopathic phenotype via induction of perivascular astrogliosis, confirming that astrocytes are major player in the cerebrovascular fibrosis (10).

Prior studies in HCHWA-D also described perivascular astrogliosis associated with arteriolar CAA (11) and perivascular ECM remodelling (12,13). Although the chronology of events is unknown, based on animal model studies, a TGFβ1-mediated fibrosis, preceding and triggering the CAA pathology might be an early event in HCHWA-D patients as well.

Accordingly, our transcriptomic study suggests an early TGFβ1-mediated fibrosis. By comparing the human transcriptome with the transcriptome of young APP-E693Q mice at the pathway level, we could identify an overlap





in the upregulation of ECM-related pathways, which is in mice prior to the onset of CAA pathology (chapter 6). Moreover, we confirmed in patient material the increase of TGFβ-induced pro-fibrotic target genes such as plasminogen activator inhibitor-1 (PAI-1), fibronectin (FN1) and collagen (Type I Col1A1 and Type III Col3A1; chapter 3). We further proposed that the accumulation of Col1 protein in the vessel walls might be prior to amyloid deposition (chapter 5), suggesting a TGFβ1-mediated fibrosis triggering the CAA pathology.

#### *Secondary vascular remodelling*

Prolonged TGFβ1 overexpression in astrocytic-TGFβ1 mice results in degenerative changes in microvascular cells (at 9-18 month) and cerebral microhemorrhages at older age (18-24 month). Our results show that TGFβ deregulation correlates with disease severity. As most severe cases are in general the oldest patients, it suggests that also in patients TGFβ activation is prolonged, and is likely modulating the CAA pathology. The most severe cases also present with secondary vascular remodeling such as vascular calcifications studied in this thesis. Interestingly, in the neuronal-TGFβ1 mice, a microarray analysis of cortical gene expression showed upregulation of OPN as well as various genes also involved in tissue mineralization and vascular calcification, strengthening the influence of TGFβ1 on this type of vasculopathy.

### Effect of TGFβ on microglia / macrophages phenotype

TGFβ is also a major player in the inflammatory phenotype of brain microglia and macrophages. The inflammatory response can present either two or three states: a pro-inflammatory state (M1) and a repair/resolution state (M2), which is sometimes subdivided into an alternative inflammation state (phagocytic, fibrotic) or an acquired deactivation state (immunosuppressive) (14). The M2 state is associated with, and induced by, a TGFβ1 increase (15). Although not extensively studied in this thesis, the immune response in HCHWA-D is thought to be influenced by TGFβ1 upregulation and to modulate the phenotype.

TGFβ-promoted parenchymal clearance of Aβ by microglia activation is a probable underlying mechanism in HCHWA-D. Previous neuropathological studies in HCHWA-D described a tendency for a disappearance of parenchymal amyloid plaques with age (16) and identified microglial phagocytosis of non-fibrillar Aβ (11). In APP-overexpressing mice, in parallel to the increase of CAA pathology, additional TGFβ1 overexpression resulted in a microglial activation promoting the clearance of Aβ and reducing the amount of Aβ parenchymal plaques. It is suggested that TGFβ1 induces microglial phagocytosis (8) which is characteristic of M2 inflammatory

state. In HCHWA-D patients, typical M2 genes Sphk1, TGFβ1 and HO-1 (acquired deactivation) together with chitinase-Like Proteins genes CHI3L2 and CHI3L1 (alternative inflammatory, (14)) were significantly increased in our RNAseq study (chapter 6, supplementary tables).

Lastly, inflammatory CAA can also be induced by TGFβ pathway activation. Inflammatory CAA is characterized by the infiltration of macrophages and other blood-derived immune cells around and/or within the vessel walls of CAA-affected blood vessels. Perivascular macrophages have a function in the regulation of CAA (17) and TGFβ1 is a central player in the modulation of the phenotype and brain infiltration of macrophages. Indeed, in APP and TGFβ1-overexpressing mice, induction of inflammatory CAA via activation of macrophages reduces the CAA pathology and is thus considered as beneficial (18,19). At histopathological examination, inflammatory CAA is common in HCHWA-D and is more frequently detected in patients with the most severe phenotype (20). Whether inflammatory CAA is beneficial or detrimental to the patients is a matter of debate. Although it is associated with diverse vasculopathies and increased risk of dementia (16), patients with inflammatory CAA survive on average longer and have smaller non-fatal hemorrhages (M. Maat Schieman, unpublished work). In conclusion, the exact contribution of these immune cells to the CAA pathology is unclear, limited to animal models studies and requires further investigations.

- The TGFβ pathway is activated in HCHWA-D, and contributes to the phenotypic variability
- TGFβ chronic overexpression aggravates the vascular fibrosis, is associated with the CAA pathology severity and might promote vascular calcification

## Possible causes of TGFβ activation in HCHWA-D

TGFβ in the central nervous system (CNS) is induced upon injuries as part of the healing process. Independently of stroke-related brain injuries, we discuss below potential early TGFβ induction in HCHWA-D triggered by cellular stress or interference with TGFβ related processes.

### Early response to cellular toxicity

Oligomeric Aβ species (oAβs) have been extensively studied and are the most toxic of the Aβ species accumulating in AD and in APP-overexpressing animal models. oAβ are potent neurotoxins inducing a wide range of cellular





dysfunctions (oxidative and ER stress, calcium dyshomeostasis, cell cycle re-entry, mitochondrial dysfunction) as well as neurodegeneratives reactions (21,22).

A higher stability of oAβs due to the E22Q mutation (chapter 1) have been predicted *in silico* (23), demonstrated *in vitro* (24–26) and further found *in vivo*, in APP-E693Q mice (27). In APP-E693Δ mice, a closely related animal model with a lack of glutamate at position 22 of Aβ , intraneuronal oAβs lead to mitochondrial dysfunction (28). Even though the actual existence of oAβs has never been proven in patients, we also found a mitochondrial dysfunction in our HCHWA-D transcriptomic study (chapter 6) indicating cellular stress, possibly triggered by toxic oligomeric species.

While less investigated, oAβs can elicit a stress response in other cell types than neurons. Particularly, endothelial cells (ECs) *in vitro* are highly susceptible to Dutch oAβ peptide (29) and endothelial injuries lead to a CAA phenotype associated with TGFβ1 upregulation (30). Nevertheless Dutch oAβ addition to ECs in monoculture leads to a decrease in TGFβ1 (31,32), and it is likely that the TGFβ response observed *in vivo* mostly comes from other cells type such as glial cells (33). Especially reactive astrocytes are susceptible to oAβs toxic species and can induce a neuroprotective TGFβ response. A recent study revealed that oAβ can activate astrocytes; astrocyte-derived TGFβ1 prevents synapse loss induced by oAβ in AD mice (34). Such a protective astrocytic reaction to limit the level of neurotoxic soluble oAβ was also suggested to occur in HCHWA-D (11).

### Direct interferences with TGFβ activity

Finally, the Dutch mutation could directly interfere with TGFβ-related processes.

TGFβ1 co-aggregates with amyloid parenchymal plaques in AD and Down Syndrome, suggesting a neuroprotective Aβ sequestration (35). It was demonstrated that Aβ can bind to TGFβ (36) and to TGFβ receptors (TGFβR) (37). Although the effect of the Dutch Aβ peptide on the interaction with TGFβ is unknown, the FAQD Dutch Aβ motif was predicted to influence the Aβ binding to TGFβ receptors, meaning a putative direct interference with the signalling pathway.

Furthermore, Amyloid Precursor Protein (APP) and TGFβ1 play a role in the coagulation cascade. APP is able to form complex with coagulation factors *in vivo,* and in blood of HCHWA-D patients the complex APP-factors XIa was slightly increased (38). Although the exact impact of the Dutch mutation on the coagulation cascade remains to be explored, APP is accumulating in HCHWA-D vascular amyloid (39) and might be blood-derived from platelets, which are rich in APP proteins. In transgenic CAA mouse models, platelet recruitment to vascular amyloid is prolonged (in

APP23 and APP Dutch mice (40)). Since TGFβ1 is also stored in large amount in platelets and Col1 particularly can induce platelet exocytosis (41), the presence of vascular Col1 in HCHWA-D (chapter 5) might enhance platelet recruitment and exocytosis, thereby providing local higher TGFβ1 and APP concentrations and further aggravating (or even initiating) the CAA pathology.

- Pathomechanisms such as enhanced formation of oAβ, endothelial injuries or coagulation disorders could enhance endogenous TGFβ response before stroke onset

## Future perpectives

### *Pathomechanisms in HCHWA-D*

A major question raised by this work is whether the Dutch mutation itself triggers an endogenous TGFβ increase.

As described above, TGFβ1 cerebral overexpression in AD mice induces a CAA pathology increase and a parenchymal amyloid reduction. Intriguingly, a CAA phenotype is consistently induced in transgenic animals upon insertion of Dutch mutation in the APP sequence (APP Dutch (42), APP Dutch/ APP23 (43); APP Dutch/BACE1 (44); APP-E693Q/PS1Δexon9 (27); Knock-In model APP DSL (45)). TGFβ1 levels in those models would be interesting to measure in order to know whether the insertion of the Dutch mutation leads to an endogenous TGFβ1 increase as proposed in this thesis.

The direct effect of the Dutch Aβ peptide on TGFβ-related mechanisms could be further tested experimentally. As described in this chapter, some biological processes might only be triggered by specific Aβ species such as oAβs. Obtaining specific Aβ species *in vitro* using synthetic Aβ is problematic because Aβ peptide is highly unstable (personal observations). Instead, endogenous Aβ produced by iPS patient-derived cells, combined with recent progress in organoids or organ-on-chip technologies, opens new possibilities. Once established, an endogenous TGFβ increase, for example, in patient-derived astrocyte/neuronal cocultures would be an interesting phenotype for further studies into pathomechanisms or therapeutic targets.

### *Biomarkers of cerebrovascular fibrosis*

New biomarkers of CAA pathology in blood and cerebrospinal fluid (CSF) would be highly valuable to detect the disease onset, improve the clinical diagnosis and understand underlying pathomechanisms.





Results from this thesis suggest that biomarkers of cerebrovascular fibrosis such as direct measure of TGFβ levels or detection of downstream pro-fibrotic components (such as FN1, Serpine1 and OPN) could be of interest. Brain TGFβ levels in asymptomatic mutation carriers and in post-stroke patients have not been determined to date. In mutation carriers, detection of TGFβ levels might indicate the onset of brain injuries (see **Graphic summary**) and help in defining windows for therapeutic intervention. For clinical practice, biomarkers of cerebrovascular fibrosis could help to estimate disease severity and progression. Finally, monitoring such biomarkers in therapeutic intervention studies will add insight into safety, effect and effectiveness of drugs in clinical trials.

Developing biomarkers of cerebrovascular fibrosis would help to better estimate the onset and progression of CAA pathology in HCHWA-D but also in other CAA-related vasculopathy and small vessel diseases where TGFβ plays a central role as well (46,47).

- Developing biomarkers of cerebrovascular fibrosis will help understanding the disease in its initial phase, improving diagnostic tools and defining the best therapeutic strategies for HCHWA-D
- Such biomarkers are of interest for other CAA pathologies

## Therapeutics intervention

### TGFβ lowering therapies

#### *TGFβ pathway is a double-edged sword*

TGF-β is key in tissue homeostasis and disruption of the TGFβ pathway has been implicated in numerous human diseases. Activation of the TGFβ pathway in systemic diseases (such as atherosclerosis) has beneficial anti-inflammatory properties as well as detrimental profibrotic effects. Similar dual effects have been observed in the CNS. In animal models, TGFβ1 increase is neuroprotective, especially acutely after injury (48–51). However, chronically high levels cause extensive cerebrovascular fibrosis as described in this thesis.

#### *Considerations with TGFβ lowering therapies*

Given the short-term beneficial effect of TGFβ on neuroprotection, TGFβ lowering therapies should be considered with care. *In vivo* pharmacological tuning of TGFβ in APP mice is feasible (52–54). In symptomatic patients, monitoring intracerebral TGFβ to prevent high chronic levels could be beneficial. Indeed patients suffering from recurrent strokes most probably have higher TGFβ levels than pre-symptomatic mutation carriers. In the

context of stroke, acute TGFβ upregulation restricts the infarct size in mouse models (49,55). However, in ageing animals, the TGFβ response is stronger and last longer (56,57) resulting in more long-term detrimental effects. Maintaining TGFβ levels within a certain range might therefore slow down CAA severity and disease progression. In practice, strategies targeting the downstream ECM modulators and pro-fibrotic genes would be a safer option than interfering with the TGFβ signaling. For example PSTG2 (COX-2) inhibitor celecoxib can lower FN1 and Col1 expression and attenuate the vessel wall thickness in stroke-prone spontaneously hypertensive rats (58).

## Amyloid lowering therapy

### *Anti-Aβ immunotherapy*

Two strategies of Aβ vaccination have been tried in AD clinical trial, either active immunization with Aβ species to trigger a host immune response, or passive immunization by direct infusion of anti-Aβ antibodies.

But although anti-Aβ immunotherapies are effective in removing parenchymal Aβ, an increase in CAA pathology is observed, implicating a TGFβ upregulation. Amyloid-related imaging abnormalities (or ARIA) in amyloid-modifying therapeutic trials are a major concern and multiple studies indicate that vascular amyloid is the common pathophysiological mechanism behind ARIA (59). At autopsy, Aβ vaccination consistently induced a removal of parenchymal plaques and an increase in amyloid vascular deposition in brains of clinical trial participants (60,61). Anti-Aβ immunotherapy in old AD transgenic mice had similar effects, with a worsening in CAA pathology and hemorrhages with longer treatment. Interestingly this phenotype is again triggered by a change in the inflammatory state characterized by TGFβ upregulation (62).

A first immunotherapy trial for probable CAA patients with Ponezumab, an antibody-targeting Aβ40, was launched by Pfizer in 2013 (NCT01821118; https://clinicaltrials.gov). After promising results in mouse models with CAA pathology (63), infusions of humanized ponezumab or placebo were evaluated for changes in occipital vascular reactivity by BOLD fMRI (primary endpoint). Although Ponezumab was well tolerated and showed drug-placebo differences, it did not meet the primary endpoint and the trial was stopped in 2016 in phase II (results not yet published; (64)). The pathological effect of this therapy is unknown with the current biomarkers and understanding of pathomechanisms. Based on the loss of cerebrovascular reactivity in the treated patients (65) one might speculate that the treatment resulted in a possible aggravation of the CAA pathology similarly to the AD clinical trial observations. In the light of this thesis and previous literature (66), the loss of cerebrovascular reactivity could be associated with an increase in TGFβ mediated vascular fibrosis.





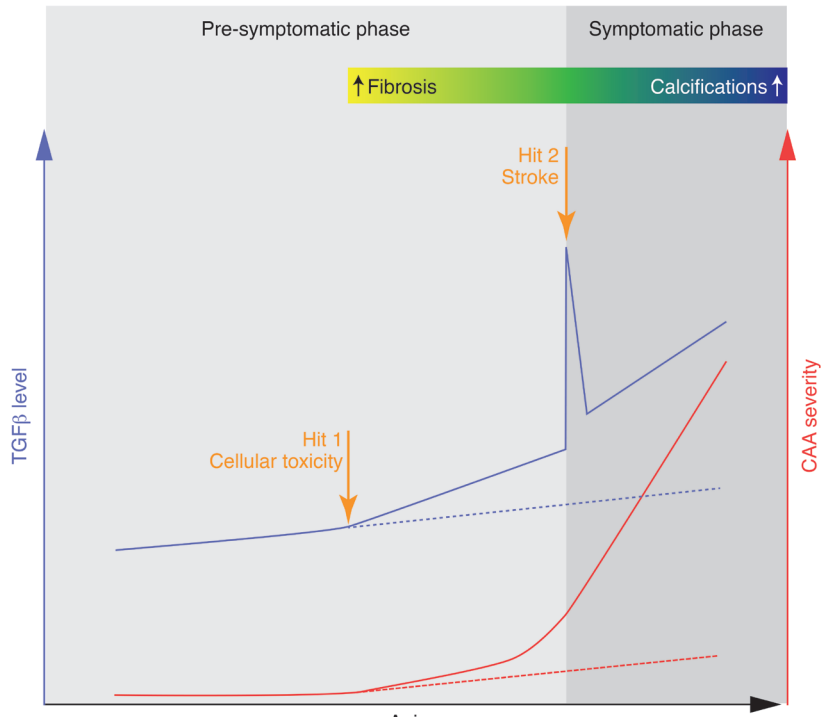
### *Other anti-Aβ strategies*

Dutch amyloid seeds are harmful and irreversible due to a prion-like effect on WT Aβ (67), therefore an early intervention is preferable. One strategy, recently described for AD (68) and currently developed in our group for HCHWA-D, uses APP exon skipping to reduce the total Aβ secretion. Targeting cerebral genes with exon skipping is feasible and already in clinical trial for Huntington's Disease, where a recent clinical trial showed very promising results (69,70).

## Alternative therapeutic strategies

Even though early anti-Aβ treatments are likely to be beneficial, therapeutic strategies uniquely focused on amyloid reduction will never completely prevent the CAA pathology in the context of vascular fibrosis. The complexity of CAA pathologies when amyloid deposition and cerebrovascular fibrosis are mixed is illustrated by studies in bitransgenic APP/TGFβ (A/T) mice (71). Therapies which were beneficial in single APP or TGFβ mice have a different outcome in A/T mice because vascular impairment caused by Aβ and TGFβ are mechanistically different (72). In HCHWA-D and particularly in sporadic CAA, where ageing plays an important role, combinatorial therapeutic treatments simultaneously targeting soluble toxic amyloid species (such as Taxifolin targeting oAβs (73)) and preventing the cerebrovascular remodeling would in all likelihood be more effective than single target treatments.

- TGFβ lowering therapies in brain should be considered with care
- Treatment to reduce the cerebrovascular fibrosis would be a safer option
- Early anti-Aβ intervention is preferable




**Graphic summary:** hypothetical pathomechanisms linking brain TGFβ increase with CAA progression. An early response to cellular toxicity could initiate the TGFβ increase (Hit 1) and trigger the vascular fibrosis in the pre-symptomatic phase. Acute stroke-related TGFβ increase (Hit 2) could further accelerate the disease progression; leading in final stage to calcification of some angiopathic vessels.





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