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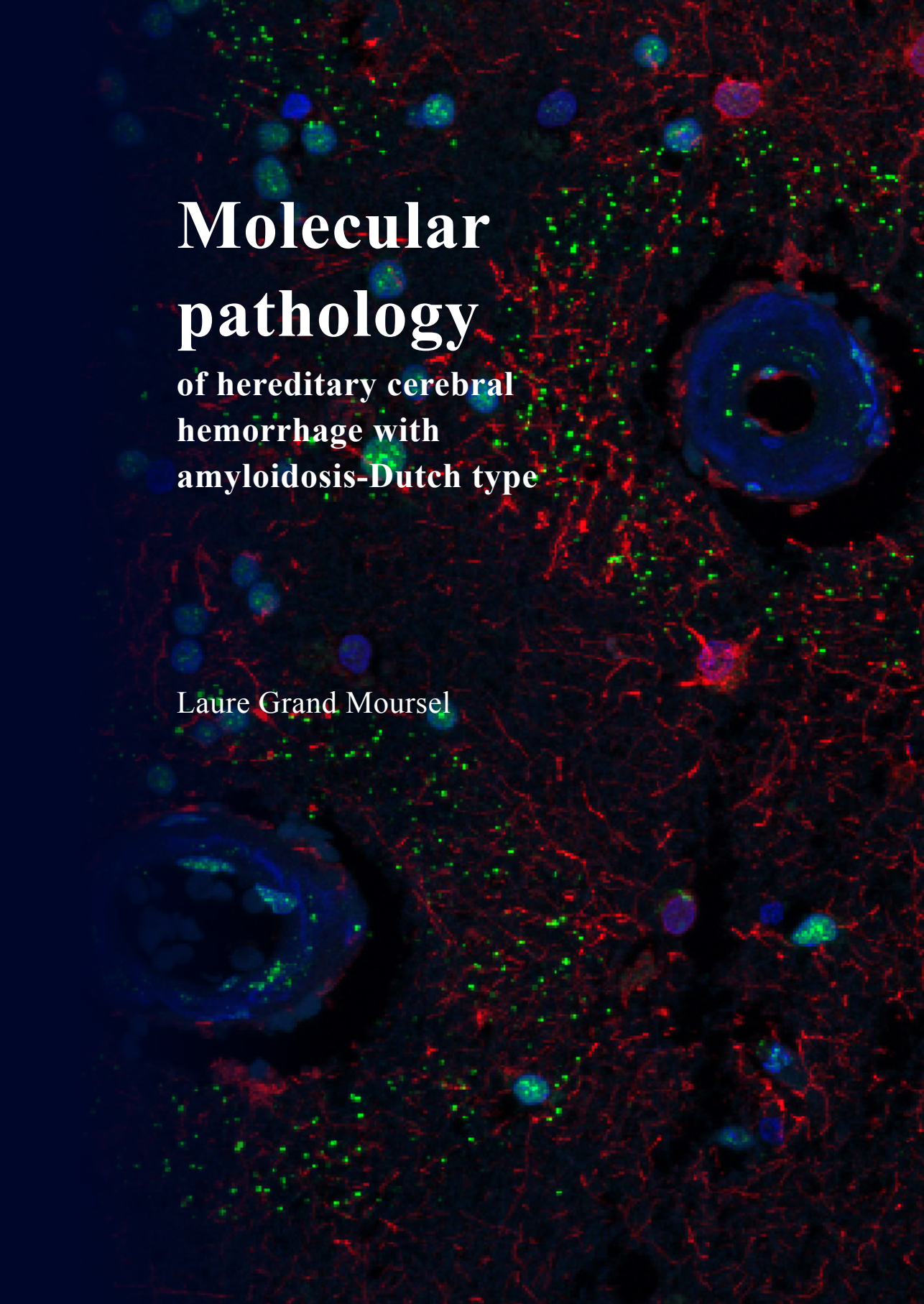## Universiteit Leiden

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Chapter 1

Introduction

Introduction

Hereditary cerebral hemorrhage with amyloidosis-Dutch type (HCHWA-D) is an autosomal dominant hereditary disease caused by a missense mutation on chromosome 21 in the amyloid precursor protein (APP), resulting in a glutamine for glutamic acid substitution (NP_000475.1:p.Glu693Gln, known as APP E693Q) (1). HCHWA-D patients suffer predominantly from hemorrhagic strokes, infarcts, and vascular dementia (2). The so-called APP ''Dutch'' mutation, originally described in families from small coastal villages in the Netherlands, is characterized by a severe Cerebral Amyloid Angiopathy (CAA) pathology.

The term CAA pathology is first used by Mandybur in 1975 (3) and is defined by the progressive accumulation of amyloid beta (Aβ) in the cerebrovasculature, resulting in acellular thickening of the vessel wall (4). CAA pathology is a risk factor for lobar intracerebral hemorrhages (ICH) (5) and for ischemic cerebral infarction (6). Because it disrupts the delicate cerebral vascular homeostasis, it can have both hemorrhagic and/or ischemic consequences (7).

HCHWA-D has been first described as the familial occurrence of ICH (8) but associated with the cerebrovascular pathology of Alzheimer's disease (AD) only twenty-four years later (9). Shortly after, the Dutch mutation is identified as the first missense mutation associated with the AD phenotype (1), due to the shared pathological brain Aβ deposition. In AD Aβ is aggregating in parenchymal plaques but CAA is also found in 80 to 90% of AD cases from autopsy series (10–17). Therefore the vasculotropic Dutch-Aβ peptide has been extensively studied to unravel the etiology of AD cerebrovascular pathology (references in this chapter).

Aβ is a 4kDa peptide resulting from APP cleavage by processing enzymes (α-and β-secretases cleaving the N-terminal peptide and γ-secretases cutting the C-terminal). Although α-secretase cleavage results in a benign pathway, Aβ peptides produced by β-secretase cleavages can aggregate and form different Aβ species ranging from monomers to fibrils. The Dutch mutation is located near the α-secretase cleavage site of APP, and is thought to affect the APP processing (18). The Aβ peptide with the Dutch mutation is also known as Aβ E22Q, as the modification occurs in position 22 (when using Aβ peptide amino acid numbering). Depending on which cleavage site is used, different Aβ peptides are formed. Most studied isoforms are Aβ (1-40) (or Aβ40, 40 amino acids) and Aβ (1-42) (or Aβ42, 42 amino acids) but many N-terminal and C-terminal truncated peptides coexists in HCHWA-D (19), adding complexity to the aggregation process of the peptides.

Clinicopathology of HCHWA-D

White matter lesions (recognized as white matter hyperintensities [WMHs] in magnetic resonance imaging [MRI] scans) are the earliest radiological manifestations in pre-symptomatic mutation carriers (detectable in standard 1.5 Tesla [T] scan). Although more subtle, the grey matter is also affected by cortical thinning in relatively young mutation carriers (mean age 46 years) when compared at 3T MRI to a control group (20). Cortical microinfarcts detected at high resolution 7T MRI are also more prevalent in pre-symptomatic patients than in controls (21).WMHs are located in subcortical areas, and tend to be more severe in the occipital lobe (22). Other radiological manifestations such as microbleeds, intracerebral hemorrhages, superficial siderosis, convexity subarachnoid hemorrhages and microinfarcts, have a higher prevalence in symptomatic patients.

Strokes in HCHWA-D are mostly hemorrhagic but ischemic strokes can also occur. The hemorrhages mostly involve temporal and occipital lobes and occur predominantly in the deeper cortical layers and in subcortical white matter (2) but can be present in the cerebral cortex as well and tend to spare the frontal lobes (23). According to the latest clinical data, the first stroke (mean age onset 54 ±8 years, n=58) is fatal in only 14% of the cases (against two- third of patients 23 years ago; (2)) and the majority of hemorrhages (35%) are located in the occipital lobe followed by the parietal lobe (21%) (24). The mean number of strokes is 3 (range 0-10 strokes) and the mean survival after the first stroke is 10 years (range 0-28 years). Epilepsy occurs in about half of the patients who have suffered from one or more strokes (25). Cognitive decline can precede the onset of hemorrhagic strokes (22) although in a recent study, cognitives abnormalities are not detected in presymptomatic mutation carriers (n=12, (21)). Cognitive decline frequently occurs in a stepwise fashion between stroke episodes (26,27).

Neuropathological features of HCHWA-D

CAA pathology in HCHWA-D is widespread and invariably affects cerebral and cerebellar meningeal arteries and cerebrocortical arterioles with a higher severity occipitally (23). The amyloid angiopathy is believed to progress from the leptomeninges towards the neocortex and in most severe cases subcortical white matter and capillaries are involved (vessels anatomy in **Figure 1**). Subcortical white matter shows areas of demyelination, axonal loss, and gliosis (leukoencephalopathy). Tau pathology (one of the neuropathological hallmarks of AD) in HCHWA-D is rare and not associated with dementia (28).

Aβ protein deposition starts at the abluminal basement membrane of vascular smooth muscle cells (VSMCs; grade 1 CAA, **Figure 2A**). At later

stages, amyloid fibrils are observed between VSMCs and in heavily affected vessels, VSMCs are completely displaced by the amyloid (grade 2 CAA, **Figure 2B**). With CAA progression, additional radial layers of Aβ (one or two) around a layer of homogeneous Aβ develop in cerebrocortical arterioles at the level of cortical layers II–III of severe cases (absent in leptomeninges and cerebellum) (23,29). Vessels with radial Aβ are associated with degenerating neurites, reactive astrocytes and activated microglia [30, Maat schieman p227].
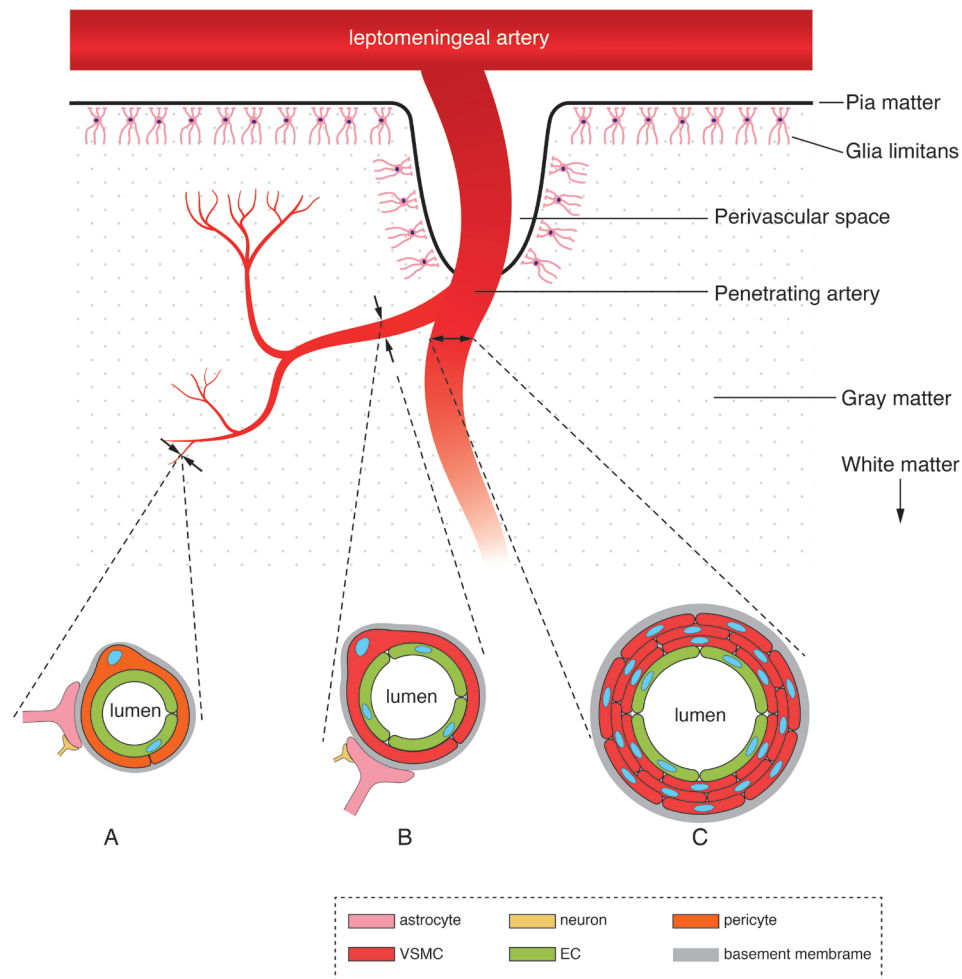

Figure 1: Illustration of the cortical brain vessels anatomy and details of the vascular components in (A) a capillary at the neurovascular unit (NVU), (B) an arteriole and (C) a penetrating artery.

Non-fibrillar Aβ clouds and fine diffuse plaques in the parenchyma tend to disappear with age and suggests a clearance of non-fibrillar parenchymal Aβ by glial cells in early stage of the disease (23,31). Indeed, although non-activated glial cells (astrocytes and microglia) are bearing cytoplasmic Aβ granules, activated glial cells in fibrillar deposits (parenchymal or perivascular associated with CAA) are devoid of Aβ granules. This suggests that glial response to fibrillar Aβ is not clearly linked with Aβ removal (32). Consequently, in contrast to parenchymal Aβ, CAA severity defined by CAA load and vessel wall thickening tends to intensify with age, even though it might differ considerably between patients of comparable age (28).

Further, disease progression in HCHWA-D is also associated with secondary microvascular degenerations or CAA-associated microvasculopathies such as vascular fibrosis (or hyalinization), microaneurysms, macrophage infiltration, "vessel-within-vessel" configurations (grade 3 CAA in **Figure 2C**), calcifications and vascular thrombi (33). CAA-associated microvasculopathies correlate with the number of cerebrovascular lesions (CAA-associated hemorrhages and/or infarcts; (34)) and with dementia diagnosis (28).
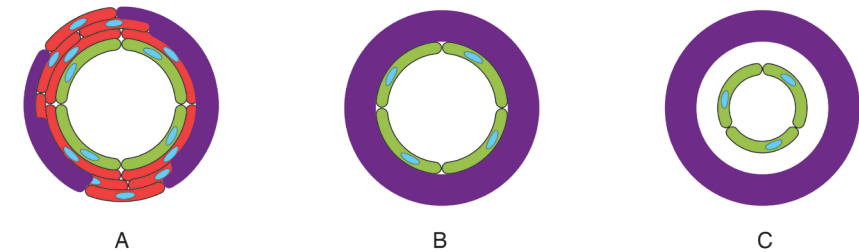
Figure 2: Illustration of CAA severity grading (based on Vonsattel & Greenberg hemorrhage risk scale 1 to 3,(106)). (A) Grade 1 CAA. Start of amyloid deposits (purple colour). (B) Grade 2 CAA. The media of this cortical vessel is fully replaced by amyloid and the wall is thickened. (C) Grade 3 CAA. Example of secondary microvasculopathies classified as grade 3, here a cracking of the vessel wall that creates a "vessel-within-vessel" appearance.

Aβ-associated proteins found in vascular deposits are investigated in HCHWA-D. Similarly to findings in AD, Amyloid P-component and Apolipoprotein E (ApoE) are identified in vascular and parenchymal Aβ (35–37). Particularly, APP and CystatinC immunoreactivity are strongly associated with CAA in HCHWA-D (38,39). Inflammatory proteins are not investigated in HCHWA-D brains except for the presence of complement proteins in amorphous plaques (40) and the association of molecular chaperone proteins with CAA (HspB8, Hsp20 and Hsp27), which could

mediate inflammatory reactions (41). The occurrence of other proteins could also vary depending on the type of material used (cryosections vs formalin-fixed and antibodies specificities). As such the presence of heparan sulfate proteoglycans found by van Horssen (42) but not by van Duinen (43), α1-antichymotrypsin found by Timmers (35), but not by Rozenmuller (40) and low-density lipoprotein receptor-related protein-1 (LRP-1) found by Wilhelmus (44), but not by Deane (45) are a matter of debate.

One characteristic feature in HCHWA-D is the presence extra-cellular matrix (ECM) parenchymal coarse deposits presenting a strong immunoreactivity for several ECM components (HSPG, laminin, collagen type 3 and 4), clustering around thick-walled Aβ vessels, but not associated with Aβ plaques. Collagen type 1 and 3 and fibronectin are associated with strongly Aβ-laden arterioles (43). Interestingly, lysyl oxidase (LOX) and tissue transglutaminase (tTG), which are enzymes cross-linking ECM-proteins, are associated with angiopathic vessels in HCHWA-D (46,47). Laminin, fibronectin, HSPG, and collagen are substrate for these enzymes whose reaction might participate in CAA formation.

Pathomechanisms of HCHWA-D

In vitro studies in human cerebrovascular cells have largely demonstrated a higher toxicity of the synthetic Aβ Dutch peptide compared to the wild type peptide (48–54). More precisely, with the Dutch mutation, a charged residue is substituted with an uncharged residue that results in higher oligomer stability favorizing the formation of these non-fibrillar toxic assemblies (55–58). Moreover, Dutch amyloid seeds, have prion-like effect on WT Aβ and are thus harmful and irreversible (59).

Amyloid deposits in HCHWA-D are a mixture of wild type and Dutch variant (60,61). Whereas CAA in general is mostly composed of Aβ40 and to a less extent of Aβ42, it is hypothesized that the Dutch mutated Aβ42 might seed and trigger the CAA formation. Indeed, both Dutch-mutated Aβ40 and wild type Aβ40 are present in isolated vessels from brain tissue, but only the Dutch-mutated Aβ42, and not the wild-type Aβ42 is detected (19). Moreover, histological studies with C-terminal specific antibodies have shown that Aβ42 preceeds Aβ40 deposition in cortical arterioles (23). In addition, electronic microscopy identified non-fibrillar Aβ42 as the first species deposited in the basement membrane of capillaries (62).

The vascular pathogenicity of the Dutch mutation is also influenced by an increase in the Aβ40:Aβ42 ratio (63), as demonstrated in transgenic mice overexpressing in neurons the human APP gene with the E693Q mutation (64). This mouse model, called APP Dutch model, develops an extensive CAA pathology (around 22-25 months) composed of Aβ40 predominantly with few diffuse Aβ42 parenchymal plaques similar to the neuropathology of HCHWA-D. Increase in Aβ42 concentration by crossing with a PS45 transgenic line (familial AD-causing PS1 mutations) resulted in abundant parenchymal plaque formation at a young age, with limited CAA pathology (64).

So far, no risk factors have been linked with the clinical symptoms of HCHWA-D. In particular, the ApoE genotype (influence on CAA pathology described in the next section) and the polymorphism in the Presenilin (PS) 1 gene, both highly impacting Aβ transport and processing, have been investigated and did not show any significant association with the disease phenotype (65,66). Only gender may modulate the phenotypic expression of the mutation, indeed the mortality rate is respectively significantly higher for female but lower for individuals with a maternal transmission (67). Nevertheless the reason is unidentified and a recent study on a smaller cohort did not confirm the gender association with the mortality rate (24).

The Dutch mutation can also alter other mechanisms having influence on Aβ accumulation and clearance from interstitial cerebral fluid. It is suggested from *in vitro* studies that the mutation could enhance the resistance to proteolytic degradation (68,69) thereby reducing the Aβ turnover rate, while Aβ production rate seemed unaffected (70–73). Furthermore, altered affinity changes due to the Dutch mutation affects Aβ transport across the blood-brain-barrier (74). One explanation could be a lower affinity for LRP1 which could affect the LRP-mediated brain capillary clearance (45).

Pathomechanisms such as alteration in vessel wall integrity (VSMCs disappearance), activation of perivascular cell (astrogliosis and microglosis) and disturbances in peripheral drainage (basement membrane thickening), although more severe in HCHWA-D, are shared features of all CAA pathologies (75–78).

CAA pathology: sporadic CAA (sCAA) and AD with CAA

CAA pathology is frequent in normal ageing, may be asymptomatic in mild cases (79), but is an important cause of spontaneous lobar ICH (80). When taken at any level of severity, in population-based (>85 years) and community-based (median age 88.5) studies, neuropathological evidence of CAA are recorded in respectively 69.6% and 78.9% of autopsied cases (81,82). Earlier studies have related that CAA pathology is related to cognitive impairment and dementia in ageing (83). It is in addition recently proposed that besides being more prevalent among AD patients, CAA might induces an independent cognitive contribution to AD dementia (81,84).

CAA pathology in AD with CAA or in sCAA are intermingled disorders closely resembling at pathological investigation when solely regarding at Aβ vascular deposition (29). A distinction is made in subjects which rely both on the clinical diagnosis of dementia and on the definite neuropathologic

examination (tau pathology seen as neurofibrillary tangles) presence is a hallmark in AD). Both in AD with CAA and in sCAA, subjects can present an hemorrhagic phenotype which is related to CAA severity. Indeed, in AD with CAA, CAA pathology is predominantly mild to moderate but cases with the higher frequency of hemorrhages had also a more severe CAA (85). Likewise, in sCAA cases presenting lobar ICH, CAA pathology severity is, in general higher (86,87).

Besides ageing, which is the strongest known clinical risk factor for developing CAA pathology (88), certain genetic risk factor have been identified. As in AD, ApoE genotype is strongly associated with CAA pathology and has influence on the pathophysiology of CAA (89–91). ApoE ε2 and ApoE ε4 are related to more severe CAA but only ApoE ε4 is linked with capillary involvement (92,93). Carriers of ApoE ε2 risk are more at risk for CAA-related hemorrhages (87,91,94). Other non-ApoE gene polymorphisms have been compared in a meta-analysis and the strongest association with CAA pathology are found for the transforming growth factor β1 gene (TGFβ1), the translocase of outer mitochondrial membrane 40 gene (TOMM40) and the complement component receptor 1 gene (CR1) (95).

Interestingly, TGFβ1 expression modulates the CAA phenotype. In transgenic mice, both astrocytic or neuronal overexpression of TGFβ1 resulted in cerebral angiopathy due to an increase in vascular fibrosis (96,97). Moreover, co-overexpression of TGFβ1 and APP in mice resulted in a reduction in parenchymal Aβ plaque load with CAA increase (98). Multiple studies have shown a role for TGFβ1 in promoting APP and Aβ production by astrocytes *in vitro* (99–102) but also *in vivo* with increased endogenous APP and Aβ levels in TGFβ1-astrocytic mouse model (102). However, whether these animal models actually accumulate murine Aβ is likely mouse strain-dependant (103). In VSMCs, TGFβ1 associated to ApoE4 protein strongly increased the amount of cellular Aβ (104) and enhances Aβ internalization in a dose-dependent manner [30, Prior & Urmoneit p255].

Two different CAA grading methods, both based on Aβ staining, are primarily referred to in neuropathological studies. A first semi-quantitative approach is ranking CAA mostly based on its spreading from leptomeninges to intracortical area (105). A second approach focused on the vessel wall morphology of individual vessel, is assessing a scale ranging from 1 to 3 for hemorrhage risk (106), as illustrated in **Figure 2.** More recently, two similar approaches have been proposed for CAA pathologic assessment (16,107) in an attempt to find a consensus on a single method to assess CAA severity.

HCHWA-D: a model to study biomarkers & therapy for CAA pathology

Totally reliable and noninvasive diagnostic tests of CAA pathology as well as anti-CAA therapy do not exist. Therefore HCHWA-D, as an accepted monogenetic model of CAA pathology, is useful to develop biomarkers (either radiologic imaging or molecular signature) and to find new therapeutic targets.

Neuroimaging biomarkers of CAA pathology at MRI are developed in symptomatic (after first clinically assessed stroke) and pre-symptomatic HCHWA-D patients. In particular, the current diagnostic Boston criteria (based on the pattern of hemorrhagic lesions and superficial siderosis to asses probable CAA during life) have been evaluated and its sensitivity were improved by the inclusion of microbleed counts (108). Lately, two studies identified early imaging biomarker (before cognitive symptoms and ICH) for CAA pathology diagnosis by inclusion of presymptomatic carriers. In the first study, WMHs and microinfarcts which are ischaemic manifestations of CAA have been detected with high field (3T) and ultra-high field (7T) MRI (21). In the second study measuring regional cerebral blood flow (by Blood-Oxygen-Level Dependent functional MRI or BOLD fMRI), an altered vascular reactivity to visual stimulation in occipital lobe was detected in pre-symptomatic mutation carriers (109). More recently, a striped cortex appearance on 7T MRI (visualized as hypointense lines perpendicular to the pial surface) solely detected in 40% of symptomatic patients (n=15) has for that reason been described as a neuroimaging marker of advanced CAA pathology (110).

Fluid biomarkers, in particular Aβ, sAPP and Tau (total tau, and phosphorylated tau) protein levels in plasma and cerebrospinal fluid (CSF) are the most common studied biomarkers in amyloid beta related pathologies. In HCHWA-D, Aβ42 level in plasma are decreased (111) although no difference are perceived in sCAA (112). In CSF, pronounced decreased levels of Aβ40 and Aβ42 are measured in HCHWA-D pre-symptomatic mutation carriers (113) and less pronounced but consistent decreased levels are found in sCAA (114,115).

Lastly, other AD specific biomarkers have been tested in an HCHWA-D western Australian family. Pulpil flash response, which is linked to cholinergic deficit in AD, is reduced in mutation carriers and deserves further investigation (116). In the same study, mutation carriers tested with positron emission tomography imaging with Pittsburgh compound B (PiB-PET) showed a higher compound retention, although below the standard threshold for amyloid positivity in AD.

So far, the above mentioned biomarkers can estimate CAA pathology but none of them can precisely determine its severity, nor predict hemorrhagic

risk, nor assess with certainty the efficiency of a therapy. More specific biomarkers are highly needed in clinical trials of Aβ disease-modifying therapies (117). Although no therapy exist against CAA, besides focusing on amyloid removal therapies uniquely, alternative paths targeting aggravating factors of CAA pathology could help slowing down the CAA build up and delay the onset of symptoms.

Scope and outline of this thesis

The general aim of this thesis is to disentangle the molecular pathogenesis behind CAA formation in HCHWA-D. Since no proven therapeutic treatment exists to prevent or even delay the CAA pathology, understanding the underlying pathomechanisms in HCHWA-D is important and may lead to identification of potential therapeutic targets.

Chapter 2 review the literature on HCHWA-D pathogenesis, combining neuropathological and *in vitro* findings and focusing in particular on the role of ECM in HCHWA-D.

Former research in CAA field indicates the existence of factors able to influence Aβ to accumulate predominantly in vessels rather than in brain parenchyma, such as TGFβ. Identification of aggravating factors of CAA pathology that can interact with currently approved drugs is one area of investigation and therefore we explored in Chapter 3 TGFβ pathway activation in HCHWA-D compared to sCAA.

In order to interpret imaging findings and better evaluate the disease progression chapter 4 & 5 are dedicated to calcifications in HCHWA-D, a specific CAA-associated microvasculopathy. Chapter 4 is a histopathological study assessing the histological correlate of a recently discovered MRI radiologic finding in HCHWA-D. Chapter 5 is exploring the pathomechanisms leading to these abnormalities and tries to assess the relationship of these changes and the TGFβ findings from chapter 3.

Chapter 6 is an exploratory transcriptomic study to identify major deregulated pathways in HCHWA-D and broaden our comprehension of molecular pathogenesis.

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Chapter 2

Amyloid β in hereditary cerebral hemorrhage with amyloidosis-Dutch type

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Abstract

Hereditary cerebral haemorrhage with amyloidosis–Dutch type is an autosomal dominant hereditary disease caused by a point mutation in the amyloid precursor protein gene on chromosome 21. The mutation causes an amino acid substitution at codon 693 (E22Q), the 'Dutch mutation'. Amyloid β, the product after cleavage of the Amyloid Precursor Protein, is secreted into the extracellular space. The Dutch mutation leads to altered amyloid β cleavage and secretion, enhanced aggregation properties, higher proteolysis resistance, lowered brain efflux transporters affinity and enhanced cell surfaces binding. All this results in amyloid β accumulation in cerebral vessel walls, causing cell death and vessel wall integrity loss, making cerebral vessel walls in hereditary cerebral haemorrhage with amyloidosis–Dutch type more prone to rupture and obstruction, leading to haemorrhages and infarcts. Studying effects of altered amyloid β metabolism due to mutations like the 'Dutch' provides us with a better understanding of amyloid β toxicity, also in other amyloid β diseases like sporadic Cerebral Amyloid Angiopathy and Alzheimer's Disease.

Introduction

Hereditary cerebral haemorrhage with amyloidosis–Dutch type (HCHWA-D) is an autosomal dominant hereditary disease caused by a mutation in the Amyloid Precursor Protein (APP) gene on chromosome 21 (1). HCHWA-D patients suffer from haemorrhagic strokes, infarcts and vascular dementia (2). Life expectancy is reduced: the first stroke occurs between the ages of 40 and 65 and is fatal in two thirds of the patients (3,4). The patients that survive the first haemorrhage suffer from recurrent strokes (4).

HCHWA-D is a rare disease and has only been found in three founder families in the Dutch coastal villages of Katwijk and Scheveningen (3,4). A rough estimate is that likely 400-500 persons are at risk in multi-generational offspring families, but no clear data are available at this moment. An affected family described in Western Australia originates from Katwijk (5).

In HCHWA-D, amyloid beta (Aβ) accumulates in the cerebral vessels (cerebral amyloid angiopathy; CAA). Especially the meningeal arteries and the cerebrocortical arterioles are affected. The amount of CAA, quantified *ex vivo* using computerized morphometry, is strongly associated with the presence of dementia in HCHWA-D, and this is independent of parenchymal plaque density and age (6). CAA can also be found in at least 80% of Alzheimer's disease (AD) patients (7). However, in contrast to AD, the presence of intraneuronal neurofibrillary tangles is low in HCHWA-D and does not correlate with dementia (6).

Aβ results from a cascade of proteolytic cleavages of the APP gene product. The most common Aβ isoforms contain either 40 (Aβ-40) or 42 amino acids (Aβ-42), depending on the site of γ-secretase cleavage. In comparison with Aβ-40, Aβ-42 contains more hydrophobic residues and therefore is more prone to aggregation (8). Alternative cleavage of APP within the Aβ fragment by α-secretase prevents the formation of Aβ and leads to the release of the neuroprotective secreted APP (sAPP) (9). After processing of APP, Aβ is released into the extracellular space (10) where it can form parenchymal plaques or accumulate as vascular deposits in the cerebral vessels causing amyloid angiopathy (11). Brains of HCHWA-D patients show few parenchymal plaques, but multiple vascular Aβ deposits.

In this review, it will be described how the Aβ mutation of HCHWA-D patients modifies Aβ properties regarding aggregation, binding to cerebral vessel wall cells, interplay with extracellular matrix, proteolysis and clearance and how these altered characteristics lead to HCHWA-D pathogenesis.

Genetics of HCHWA

Three types of HCHWA are known: Dutch, Icelandic and Italian. The Icelandic type is caused by a mutation in the Cystatin C gene (*CST3*) on chromosome 20 (12). The Dutch and Italian are caused by single point mutations at the Aβ region of *APP* on chromosome 21 (1,13). There are more known mutations in the APP gene, inside and outside the Aβ region. The mutations in the Aβ region mainly lead to an AD phenotype, but a mixed pathology (AD and CAA) is also described in patients with the Flemish, Arctic, Iowa, Italian II and II mutations (**Table 1**). The above described mutations are inherited in an autosomal dominant fashion. Recessive pathogenic mutations within the Aβ region of *APP* are also known, like the Japanese mutation (a deletion of glutamine at Aβ's position 22), and the valine substitution for alanine at position 2 (14,15). The point mutation in HCHWA-D, a cytosine for guanine substitution at codon 693 of *APP*, causes an amino acid substitution of glutamine for glutamic acid at position 22 of the Aβ region of APP. The deletion of the glutamine or the substitution of the glutamine for glycine, as present in the Japanese and Arctic types, do not cause the characteristic angiopathy of HCHWA, suggesting that the exact nature of the amino acid substitution is essential for HCHWA pathogenesis. The Dutch (Glu693Gln) and Italian (Glu693Lys) amino acid substitutions lead to a change of charge at Aβ's position 22. These changes in charge specifically enable Aβ40 to bind to the surface of cerebrovascular smooth muscle cells (SMC) and form amyloid fibrils (16). Moreover, the Dutch mutation makes the Aβ peptide more resistant to neprilysin-catalysed proteolysis, probably by interfering with the peptide's backbone spatial fitting into neprilysin's catalytic pocket, thereby increasing Aβ's half-life (17).

The Dutch mutation is located near the α-secretase cleavage site of APP (**Figure 1**), which lies between the lysine at position 16 and the leucine at position 17 of Aβ (9). Patients with HCHWA-D have reduced levels of sAPP in the cerebral spinal fluid (CSF) (18), which may be caused by an altered processing of the precursor protein or alternatively by increased binding of APP to the vessel wall, as described later. Furthermore, the location of the Aβ mutation appears to influence aggregation properties. When studying the Italian, Dutch, Arctic and Iowa mutations in Aβ42 monomers, it was shown that the Italian and Dutch mutation made the Aβ42 monomer aggregate quicker than wild type Aβ42, while the Artic and Iowa mutations made the Aβ42 monomer aggregate slightly slower than wild type Aβ42 (19). This difference in aggregation was attributed to differences in helix propensities in residues 20-23 caused by the mutations: Italian and Dutch mutation increase helix propensity, while Artic and Iowa mutations slightly decrease helix propensity. It is thought that α-helical intermediates play an important role in amyloid oligomerization (19).

Position Aβ	Mutation	Phenotype	Alias
2	Ala673Thr	Not pathogenic	
2	Ala673Val*	AD	
6	His677Arg	Not pathogenic	
7	Asp678Asn	AD	
7	Aso678His[1]	Dementia + CAA	
11	Glu682Lys	AD	
16	Lys687Asp[2]	AD	
21	Ala692Gly	AD (CAA)	Flemish
22	Glu693Lys	HCHWA	Italian
22	Glu693Gln	HCHWA	Dutch
22	Glu693Gly	AD (CAA)	Arctic
22	Glu693del*	AD	Japanese
23	Asp694Asn	AD (CAA)	Iowa
34	Leu705Val	CAA	Italian II
37	Gly708	Not pathogenic	
42	Ala713Thr	AD (CAA)	Italian III
42	Ala713Val	Not pathogenic	
43	Thr714Ala	AD/Epilepsy	Iranian
43	Thr714Ile	AD	Austrian

Table 1. Mutations within the Aβ region of *APP* Aβ= Amyloid β; *= recessive mutation; AD= Alzheimer's disease; CAA= Cerebral Amyloid Angiopathy. The mutations are shown with the affected amino acid, the affected *APP* codon and, if applicable, the amino acid alteration resulting from the mutation. Mutations were found using the Alzheimer Disease & Frontotemporal Dementia Mutation Database (80) and PubMed: [1](81); [2](82).

Cerebral amyloid plaques in HCHWA-D

Aβ deposition in AD is mainly located in the brain parenchyma in the form of plaques. In HCHWA-D, Aβ deposition is mainly found in the cerebral vessel walls, but also some parenchymal Aβ deposition in the form of plaques is present, mainly in the form of 'diffuse' plaques, lacking an amyloid core as in AD (20).

Aβ peptides show different intermediate fibrillization states before plaques and vascular deposits are formed (21). The Aβ monomers are amphiphatic, with a hydrophylic N-terminal and a hydrophobic C-terminal, and are able to adopt different conformations: α-helices, β-sheets or random coils. After arrangement of dimers and trimers, unstable and toxic oligomers are formed. These oligomers contain up to 50 monomers. Subsequently, the oligomers assemble into protofibrils, which are the relatively flexible and rod-shaped precursors of the mature fibrils. Fibrils contain multiple protofibrils and are the main components of amyloid aggregates.
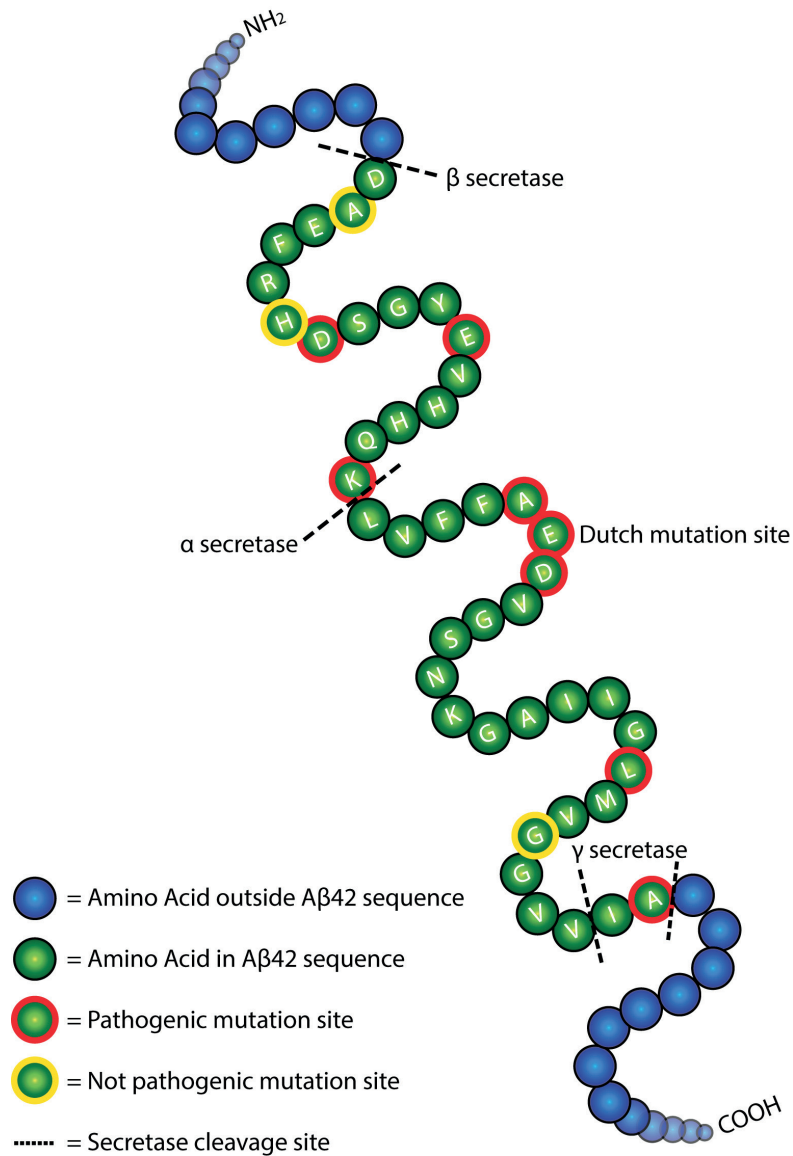
Figure 1: Location of known mutations and secretase cleavage sites in the amyloid beta sequence of the amyloid precursor protein

In the brain parenchyma, there are four different types of parenchymal plaques distinguishable in HCHWA-D: fine diffuse, dense diffuse, coarse and homogeneous. The morphology of these plaques was described by Maat-Schieman and her colleagues (20). Fine diffuse plaques are irregularly shaped, ill-defined, evenly stained, and show finely fibrous Aβ deposits. Dense diffuse plaques are either irregular, ill-defined or rounded and are stained unevenly. Coarse plaques are clusters of small, coarse and strongly staining deposits and homogeneous plaques are well-defined round shaped plaques. While all plaques show Aβ42 staining, Aβ40 staining is present in a small subset of dense diffuse and coarse plaques and in all homogeneous plaques. Only plaques containing Aβ40 harbour degenerating neurites that showed APP and ubiquitin staining. No tau is present in these degenerating neurites. In addition to the plaques, also clouds of Aβ42 were shown to be present throughout the cortex, except around Aβ containing arterioles (**Table 2**). In addition to ubiquitin, other proteins like amyloid-P, cystatin C and ApoE are known to co-aggregate with amyloid deposition in HCHWA-D (22).

In the initial stages of HCHWA-D, Aβ deposition in the form of clouds and fine diffuse plaques are present. With age, clouds disappear and plaque density increases from Aβ 40 negative fine diffuse to Aβ40 positive dense plaques (23). Electron microscopy examination showed that Aβ is non-fibrillar and plasma membrane bound initially, but when the plaques develop, amyloid fibrils accumulate (20). This development can be visualized with Congo red staining that shows increased fluorescent activity *ex vivo*. Non-fibrillar Aβ is assumed to be cleared by glial cells, thereby limiting the neurotoxic soluble form levels of Aβ in HCHWA-D patients' brains (23).

		Clouds	Plaques			
			Fine diffuse	Dense diffuse	Coarse	Homogeneous
Aβ						
	Aβ40	-	-	- to ++	- to ++	++
	Aβ42	+	+	++	++	++
DN						
	APP	-	-	- to +	- to +	±
	Ubiquitin	-	-	- to +	- to +	±
	Tau	-	-	-	-	-

Table 2. Staining intensities of parenchymal clouds and plaques Adapted from (19). Staining intensity: No staining -, few bundles ±, positive staining/small bundles + and strong positive staining/clusters ++. Aβ = Amyloid beta, DN = Degenerating Neurites.

Aβ isoforms in HCHWA-D vasculature

While Aβ42 is the main Aβ isoform in parenchymal plaques, Aβ40 is the main component of amyloid deposits in the cerebral vessels of HCHWA-D patients (24,25). Amino acid sequencing of amyloid that was isolated from leptomeningeal vascular walls showed that both mutated and wild type Aβ occurs in the vascular deposits of HCHWA-D patients (26). It has been suggested that it is especially the ratio of Aβ40 to Aβ42 that is important for vascular amyloid formation (27). Moreover, an important role for mutated Aβ42 has been proposed. In vascular amyloid of HCHWA-D, wild type and Dutch mutated Aβ40 peptides occur in a 1:1 ratio, while only the Dutch mutated Aβ42 and not the wild type Aβ42 has been detected, suggesting a possible role for Dutch mutated Aβ42 as a seed for the aggregation of Aβ40 (24). Importantly, all Aβ42 was oxidized at the methionine residue at position 35. The oxidation of Met35 of Aβ42 is known to slow down the rate of fibrillation and aggregation of Aβ42 (28). However, the Dutch mutation enables Aβ to fold into different shapes, thereby creating multiple ways to aggregate (28). In addition to Aβ40 and Aβ42, wild type Aβ37, wild type Aβ38 and Dutch mutated Aβ38 are also present in the vascular amyloid (24). Aβ37 and Aβ38 are less common isoforms of Aβ than Aβ40 and differ at the C-terminus.

It was shown that neuronal expression of APP with the Dutch mutation was sufficient to induce HCHWA pathology, i.e. CAA, smooth muscle cell degeneration and haemorrhages using a transgenic HCHWA-D mouse model. This indicates that neurons are the main source of Dutch Aβ in the cerebral vessels. Using this model it was also shown that the Dutch mutation leads to an increased Aβ40: Aβ42 ratio both in parenchymal and cerebrovascular amyloid deposits and Aβ40 was suggested to be inhibitory for parenchymal Aβ deposition (27). It was discovered in a guinea pig model of the Dutch mutation that Aβ40 accumulates around the blood vessels and in the brain due to a reduced clearance from the cerebrospinal fluid and impaired transport over the blood brain barrier, because of the lower affinity for central nervous system efflux transporters (29). Impaired clearance of Aβ was also shown in a mouse model with the Dutch and Iowa mutation: no detectable plasma Aβ but abundant Aβ deposits were present in cerebral vasculature (30). The double mutated Aβ shows a significant lower affinity to the low-density lipoprotein receptor-related protein 1 (LRP1), which is implicated in Aβ clearance, in comparison to wild type Aβ40 (31). Double mutated Aβ also seems to down-regulate LRP1 (31). However, in this model, it is not clear how each mutation affects the clearance.

In individuals with and without the Dutch mutation, Aβ40 plasma levels were similar (32). On the other hand, the plasma Aβ42 concentration of individuals with the Dutch mutation was significantly lower than in the plasma of their family members that did not carry the mutation (32). Of the 22 individuals with the Dutch mutation, 7 were still asymptomatic. Plasma concentrations of Aβ40 and Aβ42 in HCHWA-D patients did not correlate with age or severity of the symptoms (32), which indicates that plasma Aβ does not play a major role in the pathology. It is important to note that the detection method used in this study was only appropriate for soluble Aβ. Because Dutch mutated Aβ42 aggregates more readily than wild type Aβ42, the detected decline of Aβ42 in plasma of individuals with the Dutch mutation could also be due to the lack of detection of aggregated Aβ42. However, reduced Aβ42 levels as a consequence of the Dutch mutation were confirmed by *in vitro* experiments that showed a decreased Aβ42 concentration in medium of cells with the Dutch mutation, while the Aβ40 concentration was unchanged (33).

Decreased Aβ42 levels in plasma of HCHWA-D patients and in the cell model with the Dutch mutation suggest that the ratio of Aβ40:Aβ42 is elevated in HCHWA-D as compared to healthy individuals. The importance of relatively lower Aβ42 concentrations in the pathophysiology of HCHWA-D was shown in animal studies. When increasing the Aβ42 expression in transgenic HCHWA-D mice by crossing them with Aβ42 overexpressing mice, amyloid deposits were redistributed from the cerebral vessels to the parenchyma (27,32).

The nature of the mutation within Aβ was shown to be crucial in the Aβ40:Aβ42 ratio in cell models of the Flemish and Arctic mutations, where an increase in Aβ42 was present (33). Because the locations of the Dutch, Flemish and Arctic mutations are comparable (Table 1), it is not the actual mutation location but the substitution to glycine that probably affects the Aβ40:Aβ42 ratio. However, the mechanism behind this altered Aβ40:Aβ42 ratio is still unknown. Interestingly, haemorrhages are uncommon, whereas parenchymal plaques are abundant in patients with the Flemish and Arctic type mutations (34,35). This supports the role of Aβ42 in amyloid accumulation localization, as suggested in animal studies.

Aβ fibril assembly at cell surfaces

Assembly of Aβ fibrils to cell surfaces is believed to be crucial in the loss of vessel wall integrity in HCHWA-D. The assembly of Aβ fibrils has been intensively studied. Both wild type and Dutch mutated Aβ40 did not substantially assemble into fibril sheets in solution of 25 μM Aβ40, which is the Aβ peptides concentration shown to evoke pathological responses in cerebrovascular smooth muscle cells. However, at the same concentration, but in the presence of cultured cerebrovascular smooth muscle cells, Dutch mutated Aβ40 did assemble in fibrils (36). This was not the case for wild type Aβ. So Dutch mutated Aβ40 fibril formation is facilitated in the vicinity of smooth muscle cells.

After Aβ fibrillation, sAPP is able to bind to the Aβ fibrils at the smooth muscle cell surface (37). The binding of APP leads to the presence of the Kunitz-type protease inhibitor (KPI) domain, which is part of most of the APP isoforms. The KPI domain inhibits coagulant factors XIa and IXa (38), and Aβ fibrils enhance the anticoagulant property of APP (39). As a consequence, an anticoagulant environment is created, leading to an increased chance of haemorrhages. The binding of sAPP prevents its efflux from the brain, and could thus explain the reduced levels of sAPP in the CSF in HCHWA-D patients (40).

Moreover, Aβ fibrillation activates an apoptotic pathway in the cerebrovascular smooth muscle cells, leading to cell death (37). The combination of the cell death and the anticoagulant environment induced by Aβ fibrils in the vessel wall are probably major contributors to the haemorrhages in HCHWA-D patients. Also, Dutch Aβ induces increased expression and activation of matrix metalloproteinase 2 (MMP-2) in smooth muscle cells and this is believed to contribute to the Dutch Aβ-induced cell death (41). MMPs are tissue remodelling enzymes and turnover basement membranes. Elevated MMP-2 is known to lead to blood brain barrier disruption and causes cerebral haemorrhage, thus the Dutch Aβ-induced MMP-2 activation and expression probably contributes to loss of vessel wall integrity and consequent haemorrhagic stroke (41).

In addition to smooth muscle cells, pericytes are also prone to surface Dutch type Aβ fibril formation. The pericytes are even more vulnerable to the Aβ-induced degeneration compared to the smooth muscle cells (42). Pericyte degeneration was shown to be dependent on Apolipoprotein E (ApoE) genotype. ApoE is known to be the major risk factor for AD, and carrying one or two ε4 alleles is associated with a dose-dependent increase in AD risk (43).

However, in a study of 36 carriers of the Dutch mutation and 10 related controls, the ApoE ε4 genotype did not influence the age of onset of HCHWA-D, the occurrence of dementia, number of strokes nor the age at death (44). Furthermore, no association between the ApoE ε4 allele and Aβ plasma levels was found in 22 HCHWA-D patients (32). In contrast with the clinical findings, cultures of human brain pericytes with an ε4/ε4 genotype showed more Dutch Aβ-induced cell death than cultures with other ApoE genotypes (45). It is not clear what causes this inconsistency between clinical and *in vitro* studies.

In endothelial cells *in vitro*, Aβ protofibrils and fibrils induce apoptosis, and these effects are significantly stronger for Dutch mutated Aβ than wild type Aβ (46). Thus Aβ fibril formation in the vessel wall leads to an anticoagulant environment and the degeneration of three different cell types in the cerebral vessel walls, leading to CAA. This CAA leads to the haemorrhages in HCHWA-D.

The role of extracellular matrix components in cerebral amyloid angiopathy

As discussed above, reduction of Aβ clearance through the vessel wall plays a role in Aβ accumulation in the vessel wall. A major characteristic of cerebral vessels is the blood brain barrier, which prevents certain molecules to pass through the vessel into the brain and *vice versa*. Extracellular matrix (ECM) properties in the vessel wall are important for this perivascular filter by forming and maintaining basement membranes. The basement membranes are important for regulating cell growth, differentiation and migration and consist of laminins, nidogens, collagen and heparan sulphate proteoglycans (HSPGs) (47). HSPGs co-localize with the vascular deposits in AD and HCHWA-D (48). HSPGs consist of sulphated glycosaminoglycan (GAG) side chains bound to a core protein (49). Heparin and heparan sulphate are GAGs with side chains showing high Aβ affinity (50). The sulphate moieties of the side chains modulate the aggregation (51). Heparin and heparan sulphate both increase the aggregation of Aβ40 with the Dutch mutation, but especially heparin is a very potent aggregation inducer. Moreover, heparin and heparan sulphate both inhibit the cytotoxicity of cerebrovascular cells that is induced by Dutch mutated Aβ40, probably because increased aggregation prevents interactions of toxic monomeric, oligomeric or prefibrillar species of Dutch mutated Aβ40 (51). So HSPGs are modulators of Aβ aggregation and inhibitors of Dutch mutated Aβ40 cytotoxicity.

There are differences in HSPG subtype expression between AD and HCHWA-D (48) that suggest a different role for these HSPG subtypes in the different disorders. Immunohistochemical examination of AD and HCHWA-D *post mortem* brain tissue showed that the HSPG subtype agrin specifically co-localised with the vascular Aβ40 deposits in HCHWA-D, a co-localization that is less frequent in AD. In contrast, another HSPG subtype, syndecan-2 is only present in vascular deposits in AD, but not in HCHWA-D (48). These results suggest that vascular deposits in AD and HCHWA-D arise *via* different mechanisms.

Interestingly, HSPG subtypes that are usually associated with vascular basement membranes were not found in CAA, while CAA associated HSPGs syndecan-2 and glypican-1 are not expressed by vascular cells (48). This indicates that implicated HSPGs are not produced by vascular cells but have other sources and travel towards the vascular wall.

A protein that co-localizes with ECM proteins in CAA is tissue transglutaminase (tTG) (52). tTG is an enzyme involved in posttranslational modifications of proteins, like covalently cross-linked proteins (53). It plays an important role in the remodelling of the ECM after tissue injury and cell stress (54). It is known that tTG mediates Aβ40 dimerization through covalent intermolecular cross-linking and thereby seeding aggregation

(55). In early stage CAA, tTG is increased in affected vessel walls and co-localizes with Aβ deposition. This tTG could originate from endothelial cells or smooth muscle cells around which the Aβ accumulates. In later stages, co-localization is absent and tTG encloses the Aβ deposition in an abluminal and a luminal halo as shown in **Figure 2** (56). The tTG in the abluminal halo is assumed to be produced by fibroblasts in leptomeningeal vessels or astrocytes in parenchymal vessels, while tTG in the luminal halo is produced by endothelial cells in all vessel types. Moreover, ECM components fibronectin and laminin colocalize with the tTG in the halos (56). The tTGs cross-link fibronectin and laminin, and thereby stabilize the CAA. In conclusion, tTG might play an important role in the formation of vascular deposits in CAA patient.
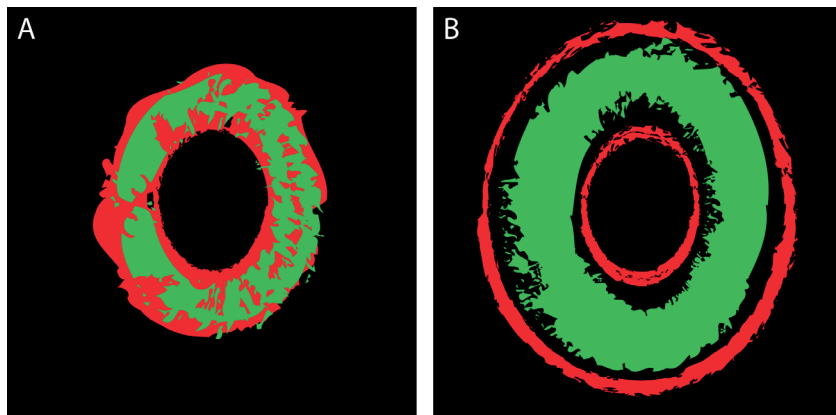

Figure 2 Schematic representation of tissue transglutaminase (tTG) and amyloid-β (Aβ) localisation in cerebral amyloid angiopathy in the neocortex of HCHWA-D patients. Aβ is shown in green, tTG in red. A. Early stage CAA: Aβ and tTG co-localize. B. Late stage CAA: Aβ and tTG do not co-localize anymore. Two halos of tTG are present: one luminal and one abluminal.

More recently, another important ECM modulator, lysyl oxidase (LOX) has been implicated in HCHWA-D and AD. LOX converts primary amines in peptide chains into aldehydes which interact to form cross-links between proteins. LOX is best known for its cross-linking of elastins and collagens in basement membranes and the ECM to maintain structural integrity (57), but HSPGs are also substrates of LOX (58). LOX is believed to play a role after tissue injury and is secreted by cells that are attracted to the brain injury sites (59). Elevated cross-linking of ECM by LOX increases permeability of the basement membrane, and thus destabilizes the vessels. LOX is present

within reactive astrocytes associated with parenchymal plaques in AD and HCHWA-D and LOX immunoreactivity is significantly increased in CAA affected vessels (58).

Potential therapies for HCHWA-D

Over the past few years, extensive research has been conducted on potential therapies for AD with the main focus on preventing formation and deposition of Aβ and tau, or increasing their clearance. Strategies reducing Aβ formation would also be interesting for HCHWA-D. Recent research has shown promising results in reducing Aβ production using RNA interference. RNA interference is a technique that down regulates gene expression by inducing degradation of targeted mRNA. Allele specific APP down regulation using short interfering RNA improved behaviour in an Alzheimer mouse model carrying the Swedish mutation (60). Using the same model, central and peripheral administration of an antisense oligonucleotide targeting APP, reduced formation of Aβ and improved the AD phenotype (61). However, APP has multiple morphoregulatory functions, like regulation of neurite outgrowth and complete knock-down of APP expression could lead to major side effects (62). Also the formation of the toxic Aβ peptides from APP could be prevented by increasing α-secretase activity or inhibiting the β- or γ-secretase activity. Epigallocatechin-gallate (EGCG), a compound that is also found in green tea, upregulates α-secretase and thereby promotes non-amyloidogenic processing of APP (63). Bryostatin 1 promotes α-secretase processing of APP by activating protein kinase C (64) and is currently in phase II clinical trials (Blanchette Rockefeller Neurosciences Institute).

Six small molecule BACE inhibitors are now tested in phase I trials (AZD3293, CTS-21166, E2609, PF-05297909 and TAK-070) and one (MK-8931) in phase II/III (65). Inhibiting γ secretase activity is not the best option, since γ secretase is involved in other pathways, like the Notch pathway (66). However, a "Notch-sparing γ secretase modulator" called Avagacestat has been tested in phase II, but led to worsening cognitive function, just like the phase III γ secretase inhibitor Semagacestat (67). Two other γ secretase targeting compounds (CHF-5074 and NIC5-15) are tested in phase II, but no results have been announced at this moment (67).

Another therapeutic agent that has been investigated for AD and could be interesting for HCHWA-D is *Scyllo*-inositol, an inhibitor of Aβ aggregation that demonstrated a decrease in CAA in an AD mice model (TgCRND8) after prophylactic administration (68). But clinical efficiency outcomes in a phase two clinical trial of AD patients using 250 mg *Scyllo*-inositol were not significantly different from placebo and higher dose studies were discontinued due to increased infections and mortalities (69).

An important feature of HCHWA-D is assembly of toxic Aβ fibrils at cell surfaces of cerebrovascular cells. The antioxidant catalase, which binds and degrades Aβ, was shown to inhibit this Aβ fibril-induced cell death in human brain pericytes (70).

The heat shock protein HspB8 could also inhibit Aβ40 accumulation at the cell surface and this reduced accumulation resulted in reduced death of cerebrovascular cells (71). This made HspB8 an interesting candidate for HCHWA-D therapy. However, more research on heat shock proteins showed that these proteins induce interleukin-6 secretion in HCHWA-D, eventually leading to an inflammatory response (72).

The endogenous bile acid Tauroursodeoxycholic acid (TUDCA) is another agent that shows therapeutic potential by preventing Aβ accumulation. Administration of TUDCA reduced amyloid deposition and prevented the defects in spatial, recognition and contextual memory in APP/PS1 mice (73) and was shown to prevent Dutch mutated Aβ-induced apoptosis of cultured cerebral endothelial cells (74).

In HCHWA-D there is a detrimental MMP-2 activation. Using MMP inhibitors, this activation can be diminished and thereby smooth muscle cell viability can be increased (41), which could lead to a lower incidence of cerebral haemorrhages.

As discussed above, ECM components play a major role in CAA. ECM modulators are therefore promising therapeutic targets for HCHWA-D. However, tTG is not a suitable target, since interfering with tTG could lead to destabilization of the vascular Aβ deposits and consequently enhance the chance for vessel wall rupture and haemorrhages. In contrast, lowering LOX activity could be an interesting therapeutic possibility, since elevated LOX activity in CAA leads to increased permeability of the basement membrane.

Immunotherapy directly targets the toxic Aβ peptides. Several vaccines have been developed for the treatment of AD, and these vaccines were promising in pre-clinical animal models. However, these vaccines did not lead to clinical improvement in several trials. This must probably be explained by the fact that in these trials participants already showed a (severe) clinical phenotype, whereas the pathogenic mechanism must already have been active for years. It is likely that individuals with 'pre-clinical' AD may benefit more from these vaccines. However, it is still a challenge to identify pre-clinical AD. This is not the case for pre-clinical HCHWA-D, because the majority of individuals with the Dutch mutation will develop symptoms of HCHWA-D.

It should be noted that because aggregated Aβ is hard to dissolve, it is better to target Aβ in the soluble state. In addition, dissolving the vascular deposits could also lead to disruption of the vessel wall, increasing the chance of haemorrhages. The clearance of soluble Aβ could be stimulated by the widely used drugs caffeine and rifampicin, since these drugs both upregulate the blood brain barrier transporter P-glycoprotein and rifampicin also upregulates LRP1 in wildtype mice (75). Since the proteolytic degradation of soluble Aβ is stimulated by ApoE, Cramer and colleagues hypothesized that enhancement of ApoE expression with the retinoid X receptor agonist bextarotene could promote Aβ clearance and microglial phagocytosis. They showed that administration of bextarotene led to a decrease in soluble and insoluble Aβ40 and Aβ42 levels, a decrease in cortical and hippocampal plaque burden and improved cognitive function of APP/PS1 mice (76). However, although the decrease in soluble Aβ was replicated (77,78), the decrease in plaque burden could not be replicated (77–80). Moreover, it is still unknown if this treatment would have an effect on CAA.

Conclusion

The Dutch mutation at position 22 of Aβ leads to multiple altered Aβ characteristics: charge alteration of the Aβ peptide leading to enhanced binding to cell surfaces and consequent Aβ accumulation, resistance to proteolysis and lowering of the affinity to brain efflux transporters.

The Dutch mutated Aβ is mainly produced in neurons, but forms fibrils at surfaces of cells in the vessel walls, where ECM modulators create an aggregation-promoting environment. The Aβ and sAPP in the vascular deposits promote cell degeneration and create an anticoagulant environment, which can eventually lead to haemorrhages.

Moreover, the elevated Aβ40: Aβ42 ratio in HCHWA-D suggests an inhibitory role for Aβ40 in parenchymal aggregation, but there is also an important role for Aβ42 as a seed for aggregation of Aβ40 in the cerebral blood vessels. Studies into HSPG subtypes suggest that vascular deposits in AD and HCHWA-D arise *via* different mechanisms.

Studying HCHWAs and their mutations provides us with a better understanding of the effects of Aβ and the differences among Aβ isoforms, which not only gives more insight in HCHWA pathogenesis, but also in other amyloidosis diseases, like sporadic CAA or AD.

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Chapter 3

TGFβ pathway deregulation and abnormal phospho-SMAD2/3 staining in hereditary cerebral hemorrhage with amyloidosis-Dutch type

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Abstract

Hereditary cerebral hemorrhage with amyloidosis-Dutch type (HCHWA-D) is an early onset hereditary form of cerebral amyloid angiopathy (CAA) pathology, caused by the E22Q mutation in the amyloid β (Aβ) peptide. Transforming Growth Factor β1 (TGFβ1) is a key player in vascular fibrosis and in the formation of angiopathic vessels in transgenic mice. Therefore we investigated whether the TGFβ pathway is involved in HCHWA-D pathogenesis in human postmortem brain tissue from frontal and occipital lobes. Components of the TGFβ pathway were analyzed with quantitative RT-PCR. TGFβ1 and TGFβ Receptor 2 (TGFBR2) gene expression levels were significantly increased in HCHWA-D in comparison to the controls, in both frontal and occipital lobes. TGFβ-induced pro-fibrotic target genes were also upregulated. We further assessed pathway activation by detecting phospho-SMAD2/3 (pSMAD2/3), a direct TGFβ down-stream signaling mediator, using immunohistochemistry. We found abnormal pSMAD2/3 granular deposits specifically on HCHWA-D angiopathic frontal and occipital vessels. We graded pSMAD2/3 accumulation in angiopathic vessels and found a positive correlation with the CAA load independent of the brain area. We also observed pSMAD2/3 granules in a halo surrounding occipital vessels, which was specific for HCHWA-D. The result of this study indicates an upregulation of TGFβ1 in HCHWA-D, as was found previously in AD with CAA pathology. We discuss the possible origins and implications of the TGFβ pathway deregulation in the microvasculature in HCHWA-D. These findings identify the TGFβ pathway as a potential biomarker of disease progression and a possible target of therapeutic intervention in HCHWA-D.

Introduction

Sporadic cerebral amyloid angiopathy (sCAA) is a disease of the elderly and is due to amyloid β (Aβ) deposition in cerebral leptomeningeal and cortical vessels and it is associated with intracerebral hemorrhages. CAA pathology is a common feature in Alzheimer's Disease (AD) and is also a defining pathological feature in hereditary cerebral hemorrhage with amyloidosis-Dutch type (HCHWA-D; (22)). HCHWA-D is caused by a Gln-to-Glu substitution at codon 693 of the Amyloid Precursor Protein (APP) gene leading to the formation of the Aβ E22Q peptide, a particularly aggregation-prone and toxic variant of the Aβ peptide (17). The Dutch mutation results in severe CAA pathology with loss of vascular smooth muscle cells and intracerebral hemorrhage typically between the ages of 40 and 65. Although the correlation between HCHWA-D carrier status, reduced cerebrovascular function and the clinical phenotype has been studied (40), the exact mechanisms underlying Aβ accumulation in the vessel wall are still largely unknown.

Some earlier studies of HCHWA-D *post mortem* brain material have focused on Aβ clearance and deposition in the vasculature by the induction and modification of extracellular matrix (ECM) proteins (39,15). Transforming Growth Factor β1 (TGFβ1) has a key role in vascular fibrosis by inducing ECM production in vessels. In postmortem AD brain material, TGFβ1 mRNA levels correlate positively with the extent of CAA pathology (46). Moreover, mouse models of TGFβ1 overexpression in astrocytes or neurons demonstrated that high TGFβ1 levels lead to vascular fibrosis (47,36). Whether these animal models actually accumulate murine Aβ in the CAA pathology-like vascular plaques remains controversial, but many *in vitro* studies have shown a role for TGFβ1 in promoting APP and Aβ production by astrocytes (12,1,21,4). Interestingly, TGFβ1 astrocytic overexpression in APP-overexpressing mice results in a CAA increase with a reduction in parenchymal Aβ plaque load (46).

TGFβ1, 2 and 3 isoforms are expressed in mammals and mediate their cellular effects through the TGFβ type I (TGFBR1) and type II (TGFBR2) receptors. TGFβ is present in an inactive form bound to the ECM and is activated by consecutive cleavage of the latent-associated-protein and pro-domain. Once activated, TGFβ binding to TGFBR2 induces transphosphorylation of the TGFBR1 kinase, which subsequently recruits and phosphorylates the receptor regulated Smad (homolog of Drosophila mothers against decapentaplegic) signal transducing proteins, SMAD2 and/or SMAD3. Phospho-SMAD2/3 (pSMAD2/3) interacts with the common Smad, SMAD4, and translocate into the nucleus to regulate target gene expression. Genes related to ECM synthesis such as plasminogen activator inhibitor-1 (PAI-1), fibronectin

(FN1) and collagen (Type I Col1A1 and Type III Col3A1 for example) are typical TGFβ target genes, and evidence is mounting that an increase in ECM production is linked to CAA pathology (14,44).

Accordingly, the aim of this study was to investigate in postmortem material whether the TGFβ pathway is involved in the pathogenesis of HCHWA-D, based on gene expression levels and histological observations. We specifically investigated in HCHWA-D if there is a correlation between the deregulation of the TGFβ pathway and the extent of CAA pathology.

Material and methods

Experimental design

HCHWA-D, healthy controls and sCAA brain material was used in the study as summarized in Table 1. Both frontal and occipital cortex were used in all studies, based on the assumption that the CAA pathology seems more severe in the occipital lobes in HCHWA-D (23) which therefore is expected to represent a more advanced disease stage compared to the frontal lobe. sCAA individuals were included to investigate whether the Dutch mutation in HCHWA-D results in a different effect on TGFβ signaling compared to CAA pathology in general. As TGFβ signaling increases with age (9), the control group was age-matched to the HCHWA-D patients. sCAA patients were significantly older (see Table 1).

We evaluated TGFβ pathway activation by immunohistochemistry in HCHWA-D brain tissue, staining for the dually phosphorylated pSMAD2 and/or pSMAD3 indicative of active TGFβ receptor signaling. These studies were performed in 11 HCHWA-D (age 60.5 years ± 10.7 years), 11 control (age 69.2 years ± 14.9 years) and 10 sCAA (age 74.8 years ± 8.0 years) cases. We also analyzed gene expression levels for several pathway components (RT-PCR, see Table 2) in a sub-set of these patients of whom frozen brain was available: 7 HCHWA-D patients (age 56.4 years ± 7.7 years) and 7 age-matched controls individuals (age 59.1 years ± 0.1 years). Frozen sCAA material was not available for this measurement.

Brain tissue

Frontal and occipital human *post mortem* brain tissue was obtained from the Netherlands Brain Bank (NBB) and from our hospital (LUMC). Written informed consent was obtained for each donor and all material and data were handled in a coded fashion maintaining patient anonymity according to Dutch national ethical guidelines (Code for Proper Secondary Use of Human Tissue, Dutch Federation of Medical Scientific Societies). The study was approved by the local Ethics Committee.

Table 1 Demographics of cases and material used in this study

Diagnosis	Source	Code	Age	Gender	PMD[a]	RTqPCR[b]	IHC[c]	IF[d]
NDC	LUMC	C1	70	M	6	X	X	X
NDC	LUMC	C9	53	F	15			X
NDC	LUMC	C10	69	M	n.a.			X
NDC	LUMC	C11	51	M	15			X
NDC	LUMC	C12	67	M	29			X
NDC	LUMC	C13	78	F	n.a.			X
NDC	NBB	C2	61	F	10	X	X	X
NDC	NBB	C3	64	F	6	X	X	X
NDC	NBB	C4	56	M	9	X	X	X
NDC	NBB	C5	55	M	8	X	X	X
NDC	NBB	C6	51	M	8	X	X	X
NDC	NBB	C7	57	F	8	X	X	X
NDC	NBB	C14	91	F	4		X	
NDC	NBB	C15	89	F	7		X	
NDC	NBB	C16	83	M	5		X	
NDC	NBB	C17	84	F	6		X	
sCAA	LUMC	S1	68	M	n.a.		X	X
sCAA	LUMC	S2	67	M	n.a.		X	X
sCAA	LUMC	S3	68	M	n.a.		X	
sCAA	LUMC	S4	78	F	n.a.		X	
sCAA	LUMC	S5	73	F	n.a.		X	
sCAA +AD	LUMC	S6	69	F	n.a.		X	
sCAA	LUMC	S7	81	M	n.a.		X	
sCAA	LUMC	S8	74	F	n.a.		X	
sCAA	LUMC	S9	84	M	n.a.		X	
sCAA	LUMC	S10	89	F	n.a.		X	
HCHWA-D	NBB	H7	71	M	6	X	X	X
HCHWA-D	NBB	H6	61	M	7	X	X	X
HCHWA-D	LUMC	H4	55	F	15	X	X	X
HCHWA-D	LUMC	H5	50	M	19	X	X	X
HCHWA-D	LUMC	H1	48	M	11	X	X	X
HCHWA-D	LUMC	H2	57	M	3	X	X	X
HCHWA-D	LUMC	H3	53	F	6	X	X	X
HCHWA-D	LUMC	H8	51	M	3		X	X
HCHWA-D	LUMC	H11	81	F	n.a.		X	
HCHWA-D	LUMC	H9	67	F	n.a.		X	
HCHWA-D	LUMC	H10	71	M	n.a.		X	

NDC non-demented control, sCAA sporadic cerebral amyloid angiopathy, HCHWA-D hereditary cerebral hemorrhage with amyloidosis-Dutch type, NBB Netherlands Brain Bank, LUMC Leiden University Medical Center, n.a. not available. HCHWA-D subjects clinical history in Supplementary Table 2

[a] Postmortem delay (in hours)

[b] Quantitative RT-PCR

[c] Immunohistochemistry for pSMAD2/3 quantification

[d] Immunohistofluorescence for double staining

Table 2 Primer list used for qRT-PCR

Genebank Acc. Num.	Name	Primers	Target
NM_000660	TGFB1	5'-TACCTGAACCCGTGTTGCTC-3' 5'-GTATCGCCAGGAATTGTTGC-3'	Intron-spanning (exons 2–3)
NM_003238	TGFB2	5'-CAATGCCAACTTCTGTGCTG-3' 5'-ATATAAGCTCAGGACCCTGCTG-3'	Intron-spanning (exon 6-7)
NM_005901	SMAD2	5'-GTTTTGAAGCCGTCTATCAGC-3' 5'-TTGTTACCGTCTGCCTTCG-3'	Intron-spanning (exon 10-11)
NM_005902	SMAD3	5'-GAAGATGGAGAAACCAGTGACC-3' 5'-ATTCGGGGATAGGTTTGGAG-3'	intron-spanning (exons 4-5)
NM_005359	SMAD4	5'-TGGAGCTCATCCTAGTAAATGTG-3' 5'-AGGAAATCCTTTCCGACCAG-3'	Intron-spanning (exon 2-3)
NM_005904	SMAD7	5'-AGGGGGAACGAATTATCTGG-3' 5'-TCGTCTTCTCCTCCCAGTATG-3'	Intron-spanning (exon 3-4)
NM_001130916	TGFBR1	5'-CGTGCTGACATCTATGCAATG-3' 5'-TCAACTGATGGGTCAGAAGG-3'	Intron-spanning (exon 7-8)
NM_001024847	TGFBR2	5'-CTGTGTCGAAAGCATGAAGG-3' 5'-AGTCAACGTCTCACACACCATC-3'	intron-spanning (exon 6-7)
NM_000602	PAI-1	5'-CAACTTGCTTGGGAAAGGAG-3' 5'-CGTCTGATTTGTGGAAGAGG-3'	Intron-spanning (exons 3–4)
NM_212482	FN1	5'-GCAGTGGCTGAAGACACAAG 5'-CCTGCCATTGTAGGTGAATG	Intron-spanning (exon 7-8)
NM_000088	Col1A1	5'- ATGACGTGATCTGTGACGAGAC 5'-TTCTTGGTCGGTGGGTGAC	Intron-spanning (exons 2–3)
NM_000090	Col3A1	5'-GACCTGAAATTCTGCCATCC 5'-GCATGTTTCCCCAGTTTCC	Intron-spanning (exons 48-49)
NM_003194	TBP	5'-CGCCGAATATAATCCCAAGC-3' 5'-GAAAATCAGTGCCGTGGTTC-3'	intron-spanning _ reference gene
NM_000983	RPL22	5'-TCGCTCACCTCCCTTTCTAA-3' 5'-TCACGGTGATCTTGCTCTTG-3'	intron-spanning _ reference gene
NM_000190	HMBS	5'-GCAACGGCGGAAGAAAA-3' 5'-CGAGGCTTTCAATGTTGCC-3'	intron-spanning _ reference gene

Quantitative RT-PCR

Frozen brain tissue was cut with a sliding microtome (Leica SM2010 R), homogenized with ceramic MagNA lyser beads (Roche) and grinded using a Bullet Blender (Next Advance). RNA was extracted immediately with Aurum Total RNA Mini Kit (Biorad), including removal of remaining genomic DNA by an on-column DNaseI treatment for 25 min. Total RNA was eluted in 60 µl of provided buffer and the RNA content was measured with Nanodrop at 260 nm. All RNA extractions were performed in duplicate and cDNA was synthesized directly after extraction with the Transcriptor First Strand cDNA Synthesis Kit (Roche) using Random Hexamer primers

at 65 °C. The cDNA was then adjusted and aliquoted at 20 ng/µL. Evaluation of RNA Integrity was performed with on-chip electrophoresis using an RNA 6000 Nano kit and a Bio-Analyzer 2100 (Agilent Technologies).

Intron-spanning primers targeting TGFβ pathway components (indicated in Table 2) were designed for qPCR using Primer3 Plus software (38). Primer pairs were first spotted into the wells (2.5 pmol of each in 2 µl). The qPCR was performed in a 384 wells plate using 6 ng of cDNA in a PCR master mix (Roche; 1 time PCR buffer with $MgCl_2$, 0.2 mM dNTPs, 0.28 U FastStart Taq DNA Polymerase) containing 1 time EvaGreen-qPCR dye (Biotum) and PCR grade water to a final volume of 8 µl per well. All samples were run in duplicate on the same plate along with three reference genes: Hydroxymethylbilane Synthase (HMBS), Ribosomal Protein L22 (RPL22) and TATA-Box Binding Protein (TBP). The amplification was performed on a LightCycler 480 (Roche) with an initial denaturation of 10 minutes at 95°C, followed by 45 cycles of 10 seconds denaturation at 95°C, 30 seconds annealing at 60°C and 20 seconds elongation at 72°C. Relative expression of the transcript levels was calculated using LinRegPCR v11.1 (32) with the raw fluorescence values as input. Transcript levels were calculated with the Geomean of the biological and technical repeat (4 points) normalized with two of the reference genes (HMBS and RLP22). The third reference gene (TBP) was used to check the normalization efficiency and the inter-plate variance. Changes in relative transcript levels were analyzed in GraphPad Prism version 6.00 using an unpaired two-sided Student's t test. Differences between groups were considered significant when $p<0.05$.

Immunohistochemical staining and quantification of pSMAD2/3 in blood vessels

Formalin-fixed, paraffin-embedded blocks of brain tissue were cut into serial 5 µm thick sections and mounted on coated glass slides (SuperFrost® Plus, VWR). Deparaffinization in xylene and rehydration through a series of ethanol concentrations were followed by antigen retrieval by cooking for 40 minutes at 0.76 bar steam pressure (Steba DD 1 ECO) in an acidic pH 6 solution (H-3300, Vector labs). Sections were then blocked for endogenous peroxidase with 3 % H_2O_2 in dH_2O for 10 minutes and for unspecific epitopes binding with blocking buffer [1 % BSA suspension in washing buffer (0.1 % Tween 20 in Phosphate Buffer Saline pH 7.4)] for 1 hour at room temperature. After, the sections were incubated with rabbit anti-pSMAD2/3 antibody (#3101S, Cell Signaling, 1:500 dilution) overnight at 4°C in the blocking buffer. Incubation with secondary anti-Rabbit HRP was followed by a DAB reaction kit (SK-4100, Vector lab) and mounting with Entellan® New (107961, Merck). Sections were scanned (Philips Ultra Fast Scanner 1.6 RA) for grading.

The grading of pSMAD2/3 staining was reproduced on scanned sections by two independent researchers (LvdG; JMdJ) blinded to the clinical diagnosis of each case. Six fields throughout gray matter areas of the slides were randomly selected at 100x magnification (2.016 mm^2 per field view). Per area, radially crosscut parenchymal angiopathic arterioles were counted. CAA load is defined here as the average number of angiopathic arterioles, identified by a typical thickened vessel wall, per mm^2. Presence of Aβ in angiopathic arterioles was checked on a consecutive slide (data not shown, not used for the grading). pSMAD2/3 deposits in the tunica media of angiopathic arterioles was determined for each vessel at 400x magnification. Difference in CAA load between frontal and occipital cortex was assessed with GraphPad Prism version 6.00 using a paired two-sided Student's t test. Differences between groups were considered significant when $p<0.05$.

Immunohistofluorescent double staining and pSMAD2/3 specificity

Formalin-fixed, paraffin-embedded frontal and occipital cortex 5 μm sections were used from patient material as specified in Table 1. Deparaffinization, antigen retrieval and blocking steps were identical to the immunohistochemical staining. Rabbit anti-pSMAD2/3 antibody (#3101S, Cell Signaling; 1:500 dilution) was incubated overnight at 4°C with mouse antibodies (references and dilutions in Supplementary Table 1). The antibodies were visualized with anti-rabbit Alexa Fluor® 488 and anti-mouse Alexa Fluor® 594 respectively (1hour at RT). Alternatively, Tyramide Signal Amplification (TSA® biotin detection kit, NEL700A001KT, Perkin Elmer) followed by streptavidin, Alexa Fluor® 488 conjugate was used when specified in the text (Supplementary Table 1). Nuclei were stained with DAPI (1 ug/mL) during the secondary antibody incubation step. After each incubation, the slides were extensively washed in washing buffer. Sections were mounted in Pro Long Diamond (Life technologies). Images of the fluorescent staining were acquired using a confocal laser-scanning microscope (Leica SP8, Leica Microsystems). The specificity of pSMAD2/3 staining was assessed either with calf intestine alkaline phosphatase treatment removing the phosphor-epitopes (adapted from (37), details in Supplementary Figure 1) and by using different phospho-antibodies for the same epitope (Supplementary Figure 1 and Supplementary Table 1). No cross-reactivity were observed with anti-pSMAD1/5/9 (data not shown). A granular perivascular pSMAD2/3 staining was also detected by Dr Ueberham.

Results

TGFβ1 and TGFBR2 are upregulated in HCHWA-D

From literature we know that the SMAD-dependent signaling TGFβ pathway is activated upon binding of TGFβ1 or TGFβ2 to TGFBR2, followed by phosphorylation of SMAD2 and 3 by TGFBR1. To assess TGFβ pathway implication we measured gene expression levels of TGFβ1, TGFβ2, TGFBR1, TGFBR2, SMAD2, SMAD3, SMAD4 and SMAD7 by qRT-PCR. The relative gene expression levels of TGFβ1 and TGFBR2 were significantly higher in the frontal and occipital lobes of HCHWA-D patients compared to age-related controls (Figure 1). TGFBR1 followed a similar trend, but did not reach statistical significance. TGFβ2 levels were significantly higher in the frontal lobe of HCHWA-D samples, especially in the two eldest patients from the gene expression study (H6 and H7); (Figure 1). Interestingly, these two samples also present the highest level of the inhibitory Smad, SMAD7, suggesting an enhanced TGFβ pathway activation compared to the other HCHWA-D patients (Supplementary Figure 2). Other signaling effectors of the canonical SMAD pathway (SMAD2, SMAD3, SMAD4 and SMAD7) were further not significantly different (Supplementary Figure 2).

Plasminogen activator inhibitor-1 (PAI-1), fibronectin1(FN1), Col1A1 and Col3A1 are known SMAD-dependent downstream targets of TGFβ. PAI-1 and FN1 gene expression level were both significantly higher in frontal and occipital cortex of HCHWA-D patients. Col3A1 and Col1A1 were upregulated in both brain area, but reach statistical significance in the frontal cortex only (Figure 2).

pSMAD2/3 is accumulating in HCHWA-D, but not in sCAA

To evaluate TGFβ pathway activation by immunohistochemistry in HCHWA-D brain tissue, we stained for the TGFβ1 down-stream signaling effector dually phosphorylated pSMAD2/3. Frontal and occipital cortex of controls, sCAA and HCHWA-D cases were assessed for pSMAD2/3 staining. In all the observed cases, pSMAD2/3 labelling was predominantly located in nuclei of neurons, with little or no cytoplasmic staining detected in both brain areas.

Both in frontal and occipital cortex, pSMAD2/3 staining in amyloid-laden vessels were found uniquely in HCHWA-D cases. The staining pattern showed distinct granules in the tunica media as well as diffuse staining covering the entire vessel wall. Examples of these granular deposits are given in Figure 3A. pSMAD2/3-positive and negative vessels were not morphologically different.. Notably, pSMAD2/3 deposits were mainly found in parenchymal arterioles with Aβ covering the entire vessel circumference (double staining with anti-Aβ antibodies, Figure 3B),

corresponding to an advanced CAA grade (grade 2 or 3 based on the Aβ content as defined by Greenberg and Vonsattel (13)). Nevertheless these granular deposits in the tunica media were not colocalized with the smooth muscle actin (SMA) staining, and we could observe in some angiopathic vessels an accumulation of pSMAD2/3 granules in vacuoles believed to be the remains of vascular smooth muscle cells (Supplementary Fig 3).
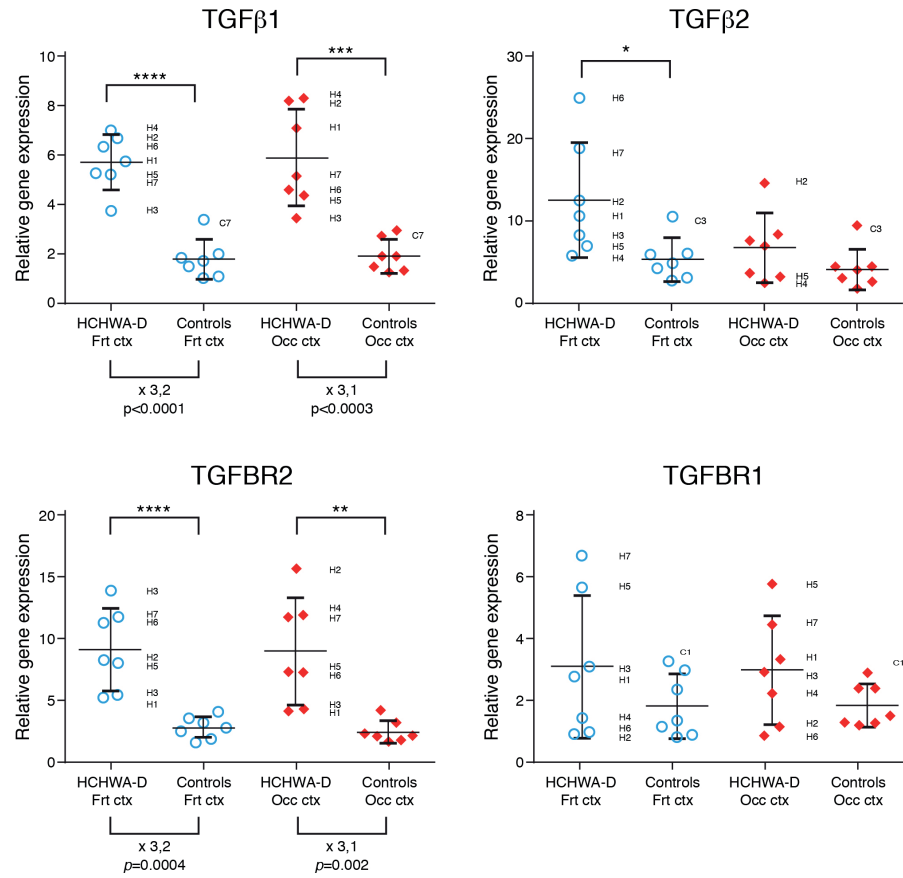
Figure 1: Significant upregulation of TGFβ1 and TGFBR2 gene levels (*left panel*; *x* indicates the time fold change) in HCHWA-D frontal (Frt) and occipital (Occ) cortex compared to age-related controls. Transcript expression levels in postmortem brain cortex were normalized with two reference genes and represented in a dot plot with mean ± SD of 7 samples; *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001 as determined by a two-tailed unpaired Student's *t* test

Figure 2: Significant upregulation of PAI-1 and FN1 gene levels (*upper panel*; *x* indicates the time fold change) in HCHWA-D frontal (Frt) and occipital (Occ) cortex compared to age-related controls. Col3A1 and Col1A1 increase reach statistical significance in frontal cortex only. Transcript expression levels in postmortem brain cortex were normalized with two reference genes and represented in a dot plot with mean ± SD of 7 samples; *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001 as determined by a two-tailed unpaired Student's *t* test
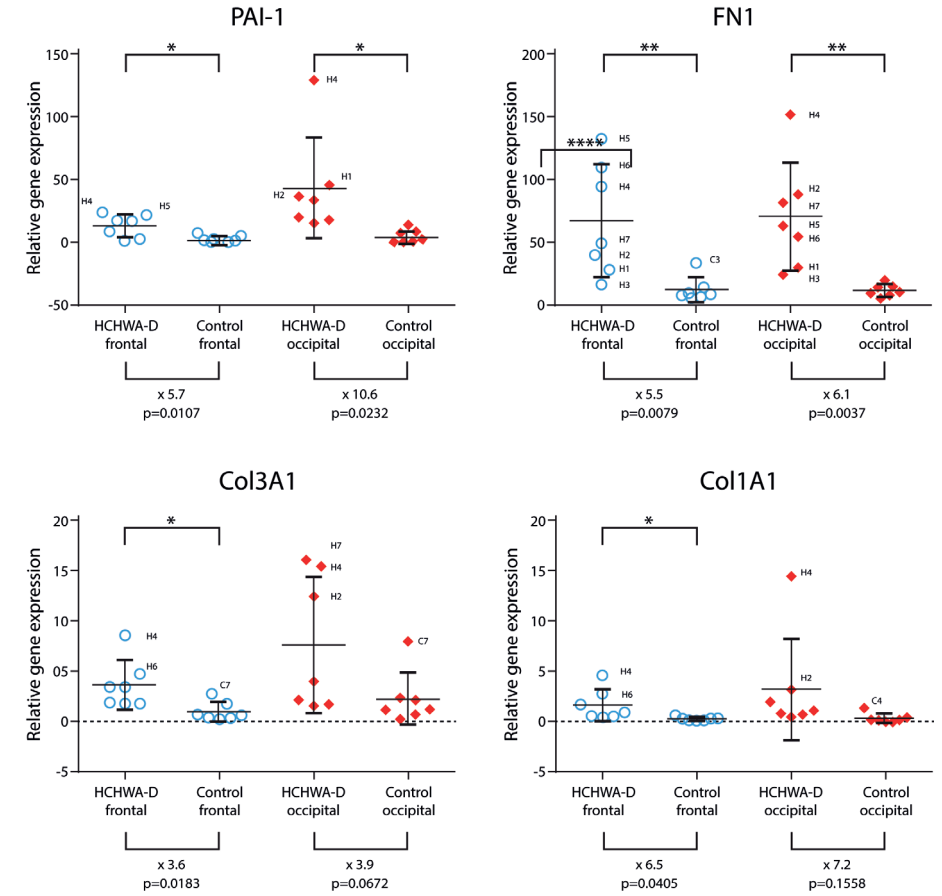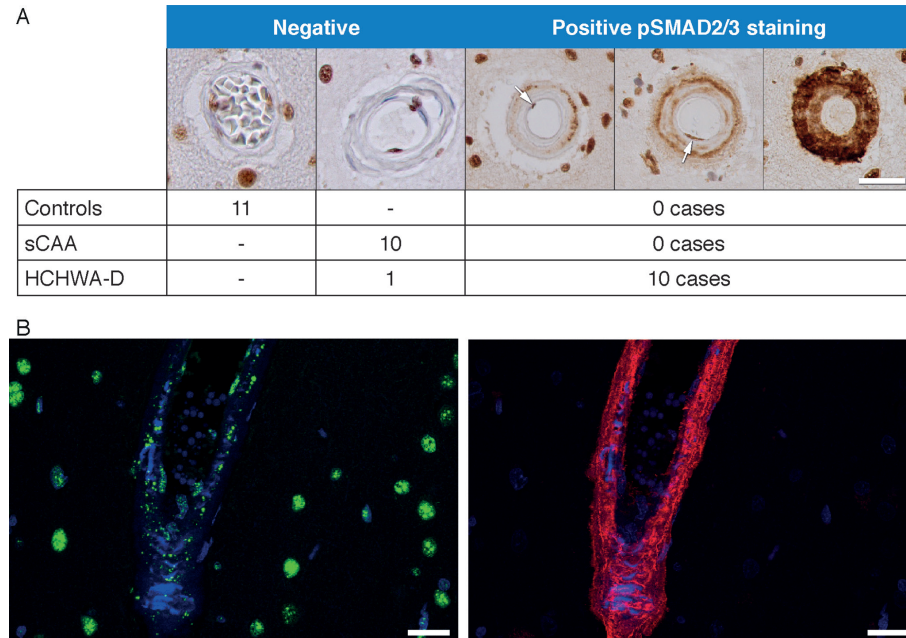
Figure 3: pSMAD2/3 granular deposits in angiopathic vessel walls. (**A**) Examples of immunohistochemical pSMAD2/3 staining graded as negative and positive. pSMAD2/3 deposits were found in 10 out of 11 HCHWA-D cases (at least one positive vessel per slide), none were found in controls or sCAA cases. Staining of endothelial cell nuclei are not counted (see the ''negative'' example). (*arrows* illustrate nuclei of endothelial cells in luminal position). (**B**) Immunohistofluorescent double staining with pSMAD2/3 TSA (*green*), Aβ 6E10 (*red*) and nuclei (*blue*). pSMAD2/3 deposits in parenchymal angiopathic arterioles, with Aβ covering the entire vessel circumference. HCHWA-D H2 patient-occipital cortex, merged confocal stack. *Scale bar* (**A**, **B**) 25μm

pSMAD2/3 accumulation is correlated with CAA load, but less with age

Entire tissue sections were graded to assess pSMAD2/3 staining in HCHWA-D samples. For the same sections, the CAA load was measured as the number of angiopathic arterioles in a defined area. We found no significant differences in CAA load between frontal and occipital cortex (Figure 4A). However, a significant positive correlation between pSMAD2/3-positive angiopathic vessels and CAA load was found independently of the brain area studied (Figure 4B). This confirmed that pSMAD2/3 deposits are only present once the CAA pathology becomes more severe. In areas with a high CAA load, most angiopathic vessels are pSMAD2/3-positive. Although there is a strong correlation between pSMAD2/3 and CAA pathology

severity, CAA load in itself is only moderately dependent on the age of the patients (Fig 4C and 4D). In particular, the 81-years-old patient, who reached an unusual age for HCHWA-D, was much less affected than expected.
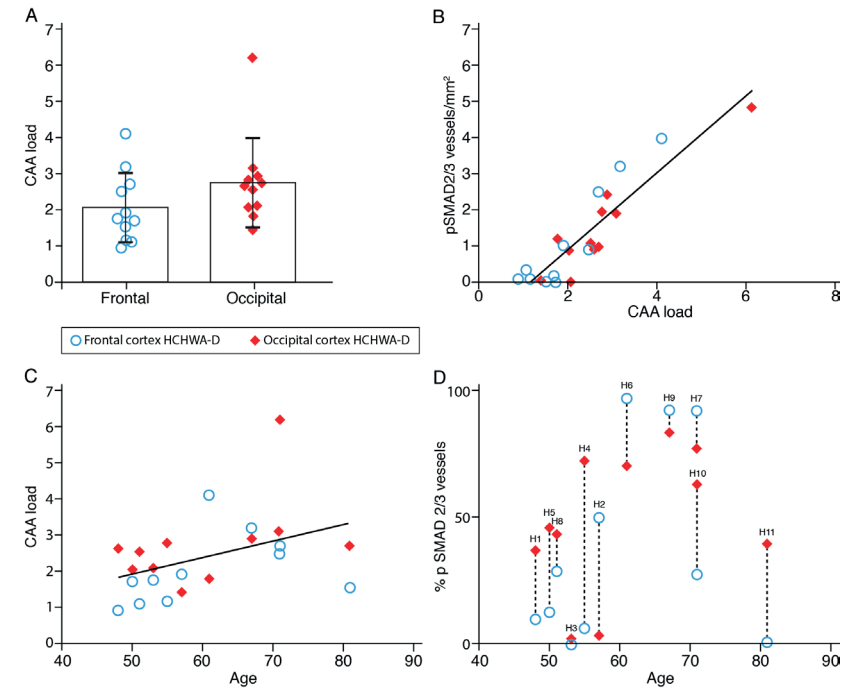
Figure 4: Quantification of CAA load and relationship with pSMAD2/3 accumulation in HCHWA-D. (**A**) CAA load was higher in occipital cortex but did not reach statistical difference (two-tailed paired Student's t test; the graph represents the mean ± SD of angiopathic vessels per mm^2). (**B**) Angiopathic pSMAD2/3 vessel wall deposits were correlated positively with CAA load (both expressed as number of vessel per mm^2; r^2=0.8220, p<0.0001). (**C**) CAA load was weakly correlated with age (r^2=0.1702; not statistically significant). (**D**) Proportion of angiopathic vessels in HCHWA-D subjects with pSMAD2/3 vessel wall deposits (*dashed lines* link brain parts of the same subject; *H numbers* refer to Table 1)

Perivascular pSMAD2/3 granules are present in occipital cortex of HCHWA-D

Apart from granules localized on angiopathic vessels in HCHWA-D, pSMAD2/3 granules were also found in the parenchyma. These were observed as perivascular parenchymal rings (see Figure 5A and B) in the occipital cortex in about 50 % of the cases (Supplementary Table 2), but

never in the frontal cortex of HCHWA-D brains or in sCAA and control cases. Perivascular rings were found both around angiopathic and non-angiopathic vessels, as well as around capillaries, and were found in clusters with a predilection for the first and last cortical layer of the gray matter. SMAD4 binding to pSMAD2/3 is a prerequisite for SMAD2/3 to enter the nucleus and initiate gene transcription. However, in our samples, SMAD4 was neither co-localized with granular vascular, nor with perivascular halo of pSMAD2/3 staining (Supplementary Figure 4); suggesting that these granules are probably not participating in the active signalling.
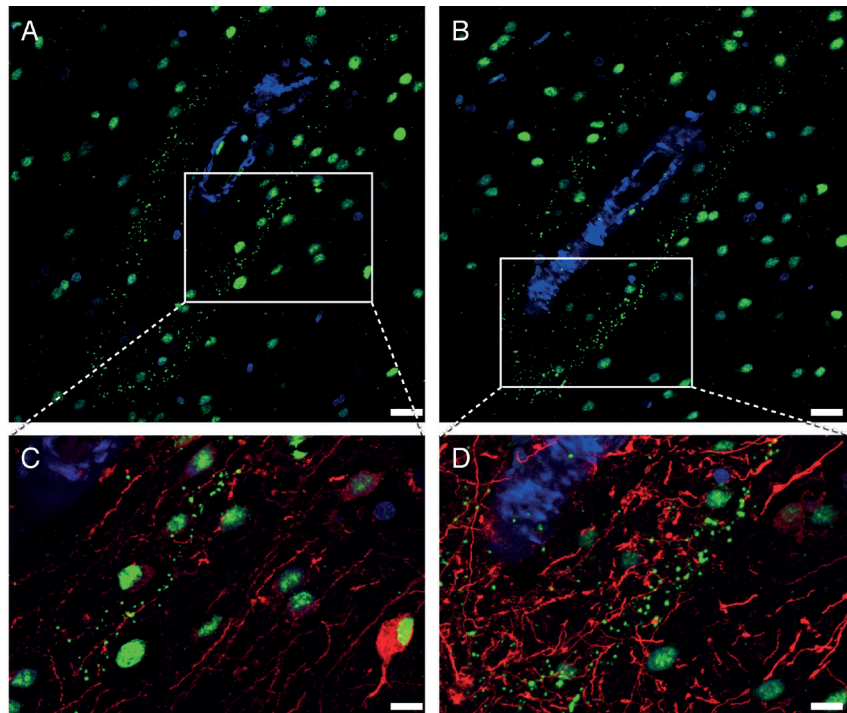
Figure 5: Perivascular pSMAD2/3 granules showed a linear alignment in a vessel with a longitudinal cut in HCHWA-D occipital cortex. Immunohistofluorescent double staining with neuronal (MAP-2) and astrocytic (GFAP) cytoskeleton markers. (**A-B**) pSMAD2/3 TSA (*green*) and nuclei (*blue*) channels. (**C-D**) detail including MAP-2 (*red*; **C**) or GFAP (*red*; **D**). Presence of perivascular pSMAD2/3 granules along the neuronal MAP-2 dendrites (**C**), and never colocalizing with GFAP processes (**D**). Consecutive sections of HCHWA-D H1 patient occipital cortex, merged confocal stack. *Scale bar* (**A-B**) 25μm; (**C-D**) 10μm
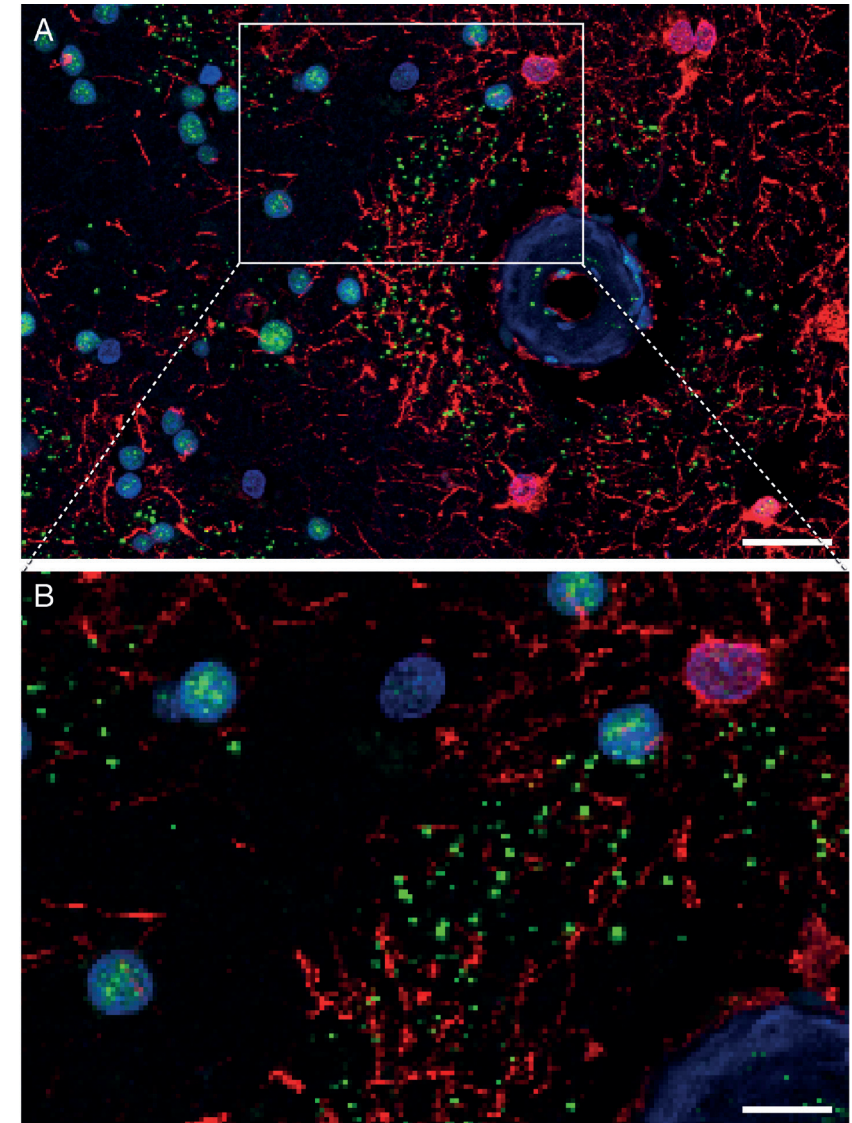
Figure 6: Perivascular ring of pSMAD2/3 granules limited to the area of GFAP-positive astrocytes. Immunohistofluorescent double staining with pSMAD2/3 (*green*), GFAP (*red*) and nuclei (*blue*). (**B**) detail of (**A**). HCHWA-D H1 patient occipital cortex, merged confocal stack. *Scale bar (***A***)* 25μm; (**B**) 10μm

Perivascular pSMAD2/3 granules do not co-localize with studied cell types or neuropathological features

Since perivascular pSMAD2/3 granules were arranged in a linear alignment, reminiscent of cytoskeletal filaments (Figure 5), we investigated whether the granules co-localized with a particular cell type using neuronal and astrocytic cytoskeleton markers. MAP-2 staining revealed a presence of the granules along perivascular dendrites (Figure 5C) without co-localization within the neuronal processes. pSMAD2/3 granules never aligned with GFAP processes (Figure 5D) but were topographically restricted to GFAP-positive perivascular areas, as depicted in Fig 5. Further, parenchymal cytoplasmic pSMAD2/3 granules were incidentally identified in neurons in some HCHWA-D individuals (Supplementary Figure 5).

Previous HCHWA-D studies reported perivascular ubiquitinated and phosphorylated neurites associated with preamyloid and amyloid deposits around angiopathic vessels (33,24). Therefore, we examined in HCHWA-D occipital samples whether the pSMAD2/3 perivascular granules were co-localized with any of these previously reported deposits. No co-localization of pSMAD2/3 granules was found in the perivascular rings with Aβ, hyperphosphorylated Tau (pTau, AT-8 antibody) and ubiquitin. Likewise, we stained for perivascular coarse deposits of ECM as described previously for HCHWA-D using collagen IV and laminin antibodies (39). Although we found these coarse deposits, they did not co-localize with pSMAD2/3 granules (Figure 7A and B). Furthermore, an extracellular pSMAD2/3 deposition with amyloid deposits and neuritic plaques was described in AD (37,6). Even though a similar co-localization with diffuse parenchymal Aβ plaques in HCHWA-D was occasionally detected, these extracellular pSMAD2/3 deposits had a fibrous-like and diffuse staining that is different from the bright round-shaped dots composing the pSMAD2/3 granular rings (Supplementary Figure 6).

Discussion

Our findings suggest that TGFβ is implicated in the pathogenesis of HCHWA-D. We found an upregulation in gene expression of several components of the TGFβ pathway and its direct downstream signaling targets as well as a strong correlation of pSMAD2/3 deposits with CAA load.

Based on previous studies in HCHWA-D describing an increased CAA pathology in the occipital cortex compared to the frontal cortex (23), we hypothesized that the CAA load and the associated vascular pathology in the occipital lobe would represent a more advanced disease stage. However, in our HCHWA-D samples, individual perforating arterioles presented a
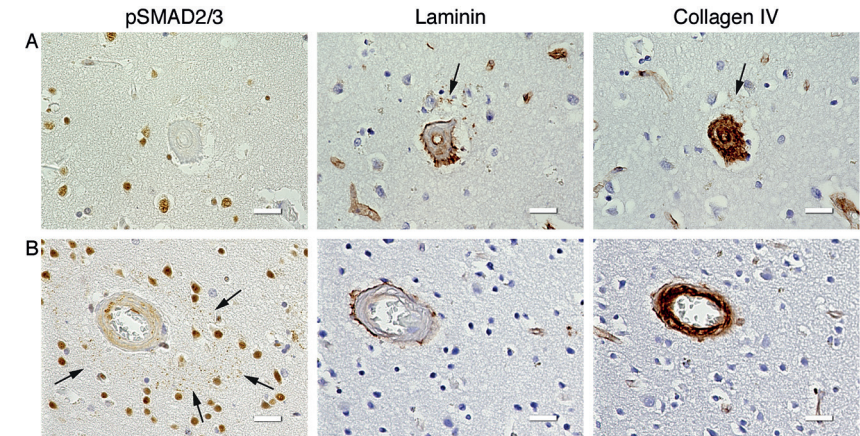

Figure 7: ECM coarse deposits did not spatially correspond to the pSMAD2/3 granules. Immunohistochemical staining of consecutive slides with pSMAD2/3, laminin and collagen IV antibodies. (**A**) Laminin coarse deposit (and weak Collagen IV colocalization; *arrows*) without presence of pSMAD2/3 granules; HCHWA-D H5 patient-occipital cortex. (**B**) pSMAD2/3 perivascular ring of granules (*arrows*) without perivascular ECM deposits; HCHWA-D H1 patient-occipital cortex. *Scale bar* 25μm

consistent moderate to severe CAA grade (grade 2 to 3), irrespective of the brain area studied. Furthermore, the CAA load, based on the number of angiopathic vessels per mm², was not significantly different in the two lobes (Figure 4A). A possible explanation for this finding is that the higher CAA load in the occipital cortex compared to the frontal cortex in previous studies is influenced by the presence of angiopathic capillaries (25). Capillary CAA is typically a feature of aged patients, while our cohort was relatively young. Additionally, the high occipital CAA load in the occipital cortex was in most cases confined to the end of the occipital horn (28), whereas our samples were obtained from diverse occipital area.

Although we did not find a difference in CAA severity between occipital and frontal lobes, we found a high correlation between the CAA load and the presence of pSMAD2/3 granules. Strikingly, these granules were only found in HCHWA-D patients, but not in sCAA cases, even in similarly affected vessels. The association of CAA pathology with pSMAD2/3, which is a direct TGFβ down-stream signaling effector, suggests an involvement of TGFβ on the vessel wall pathology. TGFβ is thought to be a key mediator of vascular remodelling (31) that is found in sporadic small-vessel diseases (34). TGFβ deregulation in the vessel wall is a central mechanism common to several hereditary brain microvasculopathies, like cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy

(CADASIL; (18)) or cerebral autosomal recessive arteriopathy with subcortical infarcts and leukoencephalopathy (CARASIL, (48,3)). TGF-β signaling was recently proposed as a common denominator of several forms of cerebral small-vessel diseases (26). Common microvasculopathies include microaneurysms, fibrinoid necrosis, obliterative intimal changes, and hyaline thickening. Interestingly, these secondary structural microvasculopathies, are found more frequently in HCHWA-D in comparison to sCAA (41) and are correlated to CAA load (28). This aggravated remodeling may be due to a fast progression of the disease in HCHWA-D or to a direct effect of Dutch-type Aβ binding to TGFBR2, directly activating the signaling pathway (16). Accumulation of pSMAD2/3 granules in the vessel wall in HCHWA-D supports the hypothesis that TGFβ deregulation contributes to secondary microvascular remodeling.

TGFβ1 was upregulated in our HCHWA-D *post mortem* brain tissue, similar to a previous study where a correlation was found in the cerebrovascular pathology of AD (45). 'Although part of the observed result is possibly influenced by differences in the cellular composition of HCHWA-D cortex, normalization per cell type cannot be achieved, nor is commonly done in RT-qPCR. We corrected for the total amount of transcript per sample with stably expressed references gene. Another potential confounder in our study is that all patients suffered one or more hemorrhagic strokes before death, which influences TGFβ1 expression. It is known that the expression of TGFβ1 increases rapidly after brain injury to restrict brain damage and as part of the healing process (11,9). Still, we found consistent upregulation in all patients in our cohort, despite very different survival times after hemorrhage (from days to several years), variable hemorrhage sites and even unrelated causes of death. This suggests that the TGFβ upregulation we found cannot solely be explained by an acute response after stroke, but is likely linked to the CAA pathology itself, as evidenced by the histological spatial correlation described above. We also found TGFBR2, the ligand-binding receptor, upregulated in HCHWA-D occipital and frontal cortex. Previous studies have also found high levels of TGFBR2 in a mouse model of traumatic cerebral injury and stroke in the chronic phase (10,30). These findings indicate that TGFBR2 upregulation is not an acute response. Lastly, the upregulation of TGFβ-induced pro-fibrotic target genes such as PAI-1, FN1, Col1A1 and Col3A1 indicate that the TGFβ signaling pathway is likely activated.

TGFβ upregulation is a double-edged sword with both protective and deleterious consequences. The vascular remodeling and fibrosis induced by TGFβ might have a protective effect in CAA pathology, due to the cross-linked increased ECM and basement membrane which might prevent the weakening of the vessel wall (49,8). Despite this protective effect in terms of stroke survival and prevention of hemorrhage, persistent TGFβ upregulation is also thought to have a major downside. The resultant ECM synthesis which modifies the composition of basement membrane impairs perivascular drainage, thereby triggering further amyloid deposition and aggravating the CAA (42,5). This was demonstrated in mouse models of both inducible neuronal- and astrocytic-TGFβ overexpression, where perivascular astrogliosis is preceding and promoting the vascular angiopathy (36,47).

In our study we found a perivascular ring of pSMAD2/3 granules around vessels in the occipital lobe of HCHWA-D samples. Comparable pSMAD2/3 granules were found in other neurodegenerative disorders, such as AD, Pick's syndrome, progressive supranuclear palsy and corticobasal degeneration (7,6,2). Typically, in AD the granules were found intracellularly and associated with neuronal aggregates of pTau or granulovacuolar inclusion of ubiquitin (37,20,2,27). This aberrant cytoplasmic dislocation of pSMAD2/3 could impair the normal TGFβ signaling pathway by sequestration of this transcription factor (37,6,2,27). In our study, we did not find pTau, ubiquitin or Aβ colocalization with the perivascular ring of pSMAD2/3 granules, which is not surprising considering the general lack of neuronal degeneration and pTau involvement in HCHWA-D. Nevertheless, the granular sequestration of pSMAD2/3 may point to a similar deregulation of the TGFβ pathway.

In the current study, we often found that the perivascular granules were positioned following a linear pattern, reminiscent of astrocytic processes. In a recent study, a decrease in cerebrovascular reactivity in the occipital cortex of HCHWA-D was described as an early biomarker of the disease (40) and astrocytes are key mediators in this process (19). In transgenic mice with cerebral angiopathy due to overexpression of TGFβ or APP, the cerebrovascular reactivity was impaired, due to neurovascular decoupling (35,29). This decoupling is the result of retraction of astrocyte end feet from the vessel wall. Similar underlying mechanisms likely occur in HCHWA-D patients. Such perivascular astrocytic remodeling has been linked in AD mouse models with CAA pathology severity and astrocytic phenotypic switch, defined by a loss of GFAP-positivity (43). The perivascular granules could be the remnants of astrocytic cytoskeletal remodeling.

In summary, our results indicate a possible contribution of TGFβ to the amyloid angiopathy and the resulting vascular remodeling seen in HCHWA-D. Future studies into the early involvement of TGFβ in the amyloid angiopathy pathogenesis should be determined in ongoing longitudinal studies in HCHWA-D.

Acknowledgments

Compliance with ethical standards

Conflicts of interest: The authors declare they have no conflicts of interest.

Ethical approval: All procedures performed in this study involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent: Informed consent was obtained from all individual participants included in the study.

Supplementary Material

The Supplementary Material for this article can be found online at: https://onlinelibrary.wiley.com/doi/full/10.1111/bpa.12533

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Chapter 4

Cerebral amyloid angiopathy with vascular iron accumulation and calcification: a high-resolution MRI-histopathology study

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Abstract

Background and Purpose: Previous studies of symptomatic and asymptomatic hereditary cerebral amyloid angiopathy (CAA) patients offered the possibility to study the radiologic manifestations of CAA in the early stages of the disease. Recently, a 'striped cortex', observable as hypointense lines perpendicular to the pial surface on T2*-weighted 7T MRI, was detected in 40% of the symptomatic hereditary CAA patients. However, the origin of these MRI contrast changes is unknown. This study aimed at defining the underlying pathology associated with the *in vivo* observed striped pattern.

Methods: Formalin-fixed *post mortem* brain material including the occipital lobe of four Hereditary Cerebral Haemorrhage with Amyloidosis-Dutch type (HCHWA-D) cases and six sporadic CAA (sCAA) cases were selected from local neuropathology tissue collections. Depending on the availability of the material, intact hemispheres or brain slabs including the occipital lobe of these patients were screened for the presence of a striped cortex. Regions containing the striped cortex were then subjected to high-resolution 7T MRI and histopathological examination.

Results: We found two HCHWA-D cases and one sCAA case with striped patterns in the occipital cortex resembling the *in vivo* signal. Histopathological examination showed that the striped pattern in the cortex at 7T MRI is due to iron accumulation and calcification of penetrating arteries. The presence of both non-heme iron and calcification on penetrating arteries causes signal loss and hence the abnormal striped patterns in the cortical ribbon on T2*-weighted MRI.

Conclusion: We identified iron accumulation and calcification of the vessel wall in HCHWA-D as the histopathological correlates of the striped cortex observed on in vivo 7T MRI.

Introduction

Hereditary Cerebral Haemorrhage with Amyloidosis-Dutch type (HCHWA-D) is a genetic disorder caused by a point mutation in the Amyloid Precursor Protein (APP; p.Glu693Gln mutation), resulting in severe cerebral amyloid angiopathy (CAA) characterized by recurrent haemorrhagic strokes with midlife onset (1, 2). Since sporadic CAA (sCAA) is also a disease of the elderly and a common feature in Alzheimer's disease, the diagnostic point mutation makes HCHWA-D a useful model to study CAA *in vivo* (2).

Previous studies of symptomatic and asymptomatic HCHWA-D mutation carriers offered the possibility to study the radiologic manifestations of CAA pathology during the course of the disease. A 'striped cortex', observable as hypointense lines perpendicular to the pial surface on T_2^*-weighted 7T MRI, was detected in 40% of the symptomatic mutation carriers, specifically in the occipital lobe (3). This pattern was only seen in advanced disease stages and not in asymptomatic patients. However, the underlying pathology of this pattern is unknown.

The feasibility of investigating histopathological correlates of MRI abnormalities have been previously shown in *post mortem* brain tissue (4-6). In the context of CAA, high-resolution *ex vivo* 7T MRI scanning allowed the detection of microvascular lesions, including microhaemorrhages, microinfarcts, and CAA-related vasculopathies (6).

The aim of this study was to define the underlying pathology associated with the *in vivo* observed striped pattern. Therefore, we screened *post mortem* brain material of four HCHWA-D donors from our local neuropathology tissue collection using 7T MRI. In two cases, striped patterns in the occipital cortex resembling the *in vivo* signal were detected. These areas were subjected to high-resolution 7T MRI to study the pattern in greater detail. Based on our hypothesis that the observed MRI contrast might be due to calcification, we added a micro computed tomography (μCT) scan as this modality is the most sensitive and specific imaging tool allowing for the identification of calcifications (7). Serial sections of the identified areas were further subjected to histological investigation to examine the histopathological correlates of the distinctive striped MRI pattern. Since HCHWA-D serves as a model for sCAA, we additionally retrospectively screened previously acquired whole hemisphere *ex vivo* 3T MRI scans of six sCAA cases. In one case (with severe CAA) a striped pattern was observed in the occipital cortex. Brain samples from that area were then subjected to 7T MRI and subsequent histopathological examination.

Material and Methods

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Study design

Formalin-fixed *post mortem* brain material including the occipital lobe of four HCHWA-D cases were selected from the neuropathology tissue collection of the Leiden University Medical Center (LUMC) in Leiden (The Netherlands) (see workflow in Fig. 1). Depending on the availability of the material, intact hemispheres or brain slabs including the occipital lobe of these patients were scanned at 7T MRI. The 7T MR images were visually inspected by two experienced observers (SvR, LvdW) and screened for a striped cortical pattern defined as hypointense lines perpendicular to the pial surface (3). Two cases showed a striped cortex on 7T MR images and smaller tissue blocks from these cases were further investigated on high-resolution 7T MRI and histology. Based on our hypothesis that the observed MRI contrast might be due to calcification, we added an μCT scan as this modality is the most sensitive and specific imaging tool allowing for the identification of calcifications (7). In addition, *ex vivo* 3T MRI scans from six sCAA cases, acquired at the Massachusetts General Hospital (MGH) in Boston (MA, USA) were retrospectively screened for the presence of a striped cortex. This pattern was observed in one sCAA case, which was then subjected to 7T MRI and histopathological examination. Case characteristics of screened HCHWA-D and sCAA cases are presented in Table 1.

Table 1: Characteristics of screened HCHWA-D and sCAA cases.

	Age	Sex	*Post mortem* delay (hours)	Striped cortex detected
HCHWA-D #1	70	Female	5	Yes
HCHWA-D #2	57	Male	2,5	Yes
HCHWA-D #3	50	Female	16	No
HCHWA-D #4	50	Male	19	No
sCAA #1	70	Male	16	Yes
sCAA #2	80	Male	Unknown	No
sCAA #3	76	Male	27	No
sCAA #4	65	Male	14	No
sCAA #5	81	Male	Unknown	No
sCAA #6	70	Female	Unknown	No

Striped cortex in vivo in symptomatic HCHWA-D patients observed at 7T MRI[3]
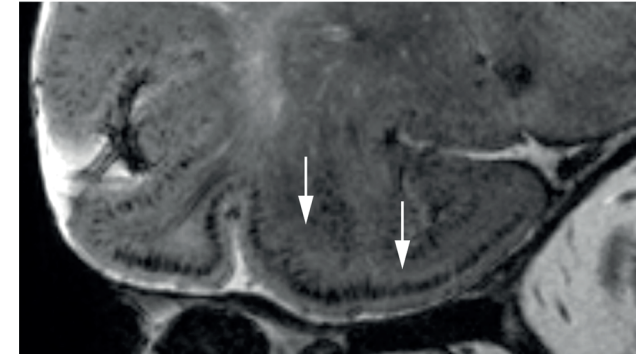
Step 1: Detection of the striped cortex on *ex vivo* MRI

Post mortem formalin-fixed hemispheres/brain slabs of HCHWA-D (n=4) cases stored in the local neuropathology tissue collection were scanned and screened

Step 2: High-resolution 7T MRI and *μ*CT of positive cases (HCHWA-D 2 cases, sCAA 1 case)

A small tissue block corresponding to a striped pattern area was resected and imaged with high resolution 7T MRI and CT

Step 3: Investigation of the histopathological correlates of the cortical changes detected at 7T MRI

Tissue blocks from step 2 were paraffin embedded. Tissue sectioning was guided by the *post mortem* MRI and based on anatomical landmarks such as the grey-white matter border, vessels, and the orientation of the striped pattern.

Histopathological assessment included staining for: iron, calcification, Aß, H&E

Figure 1: Study design and flowchart.

Brain samples and preparation

Formalin-fixed intact hemispheres or brain slabs of HCHWA-D patients were obtained from the local neuropathology tissue collection of the LUMC. Written informed consent for the HCHWA-D patients was obtained for each donor and all material and data were handled in a coded fashion maintaining patient anonymity according to Dutch national ethical guidelines (Code for Proper Secondary Use of Human Tissue, Dutch Federation of Medical Scientific Societies). Formalin-fixed intact hemispheres of the sCAA cases were obtained through an ongoing *post mortem* brain MRI study for the evaluation of MRI markers in the context of sporadic CAA at the MGH in Boston (MA, USA). Informed consent was obtained from a legal

representative prior to brain autopsy. This study was approved by the local Ethics Committees of the respective institutions.

Before MRI, the formalin-fixed brain material of the HCHWA-D patients was washed with phosphate buffered saline (PBS) for 24 h to partially restore the relaxation parameters (8). The hemisphere or brain slabs were placed in a plastic bag containing a proton-free fluid (Fomblin®, LC08, Solvay). To minimize the amount of trapped air bubbles, a vacuum was applied overnight. Before scanning, the plastic bag was sealed and fixed on a plastic plateau or using foam padding in the coil.

After scanning the intact hemisphere and brain slabs, the scans were inspected for the presence of a striped cortex by two experienced observers (SvR, LvdW). If positive, a small tissue block of approximately 20x15x15 mm was resected from the area containing the striped cortex and placed in a regular 15 ml tube (Greiner Bio-One). Before MRI, the tissue block was washed with PBS (for 24 h) to partially restore the relaxation parameters (8). Next, the 15 ml tube containing the tissue block was filled with Fomblin® (LC08, Solvay). Care was taken to avoid trapped air bubbles.

Post mortem MRI acquisition

The HCHWA-D hemispheres were scanned at the LUMC (Leiden, the Netherlands) on a whole-body human 7T MR system (Philips Healthcare, Best, the Netherlands) using a quadrature transmit and 32-channel receiver head coil. The hemisphere was positioned with the frontal lobe in the head direction and the occipital lobe in the feet direction of the scanner. A 2D T_2^*-weighted gradient echo scan was acquired with a total imaging duration of 13 minutes. Imaging parameters were: repetition time (TR)/echo time (TE) 3146/25 ms, flip angle 60°, slice thickness 1.0 mm with a 1.1 mm interslice gap and a spatial resolution of 0.21x0.21 mm.

The HCHWA-D brain slabs and smaller tissue blocks were also scanned at the LUMC (Leiden, the Netherlands), but on a 7T horizontal bore Bruker MRI system equipped with a 23 mm receiver coil (Bruker Biospin, Ettlingen, Germany). The smaller tissue blocks were scanned with a Multiple Gradient Echo (MGE) sequence using the following parameters: (TR)/(TE) 75/12.5, 23.2, 33.9 and 44.6 ms, flip angle 25°, at 100 µm isotropic resolution with 20 signal averages.

Brain slabs of one sCAA case that demonstrated a striped pattern on previously acquired whole hemisphere *ex vivo* 3T MRI scans, were subsequently scanned at 7T MRI. Scanning was performed at the Athinoula A. Martinos Center for Biomedical Imaging (MGH, Charlestown, MA, USA), on a whole-body human 7T MR Siemens MAGNETOM scanner, using a custom built 32-channel head coil, as described previously (6).

Slabs were stacked on top of each other in a glass beaker, filled with 10% formalin. A multi-echo T_2^*-weighted MRI scan was acquired using the following parameters: one run each of three flip angles (10°, 20°, and 30°), TE = 8, 18, 28, and 38 ms, at 200 µm isotropic resolution.

The most similar MRI slice with respect to the given histology was selected based on the physical location of the section in the tissue block, measured by counting the number of sections taken starting at the block surface. At this approximate location, the most similar MRI slice was chosen by visual comparing clearly detectable landmarks (contours, vasculature, tears etc.) allowing comparison of the MRI with histology.

Post mortem µCT

For the HCHWA-D cases, MRI acquisition was followed by µCT to detect the presence of calcifications. The 15 ml tube containing the tissue block in Fomblin was imaged on a Skyscan 1076 *in vivo* µCT (Bruker Biospin, Ettlingen, Germany) using the following settings: 40-48 kV, 200-250 µA, 0.5 mm filter, 2-10 averages. The same approach as described above for the MRI slice selection was used to select the most similar µCT image.

Histology and immunohistochemistry

The resected tissue blocks used for both MRI and µCT were next subjected to detailed histopathologic examination. Each tissue block was embedded in paraffin, and subsequently cut in 5 µm-thick serial sections on a microtome. The sectioning was guided by the *post mortem* MRI scans based on anatomical landmarks such as the grey-white matter border, vessels, and the orientation of the striped pattern.

The first section was stained for Aβ (anti-human Aβ, 6F/3D DakoCytomation, Glostrup, Denmark): endogenous peroxidase activity was blocked with 0.3% H_2O_2 in methanol followed by an antigen retrieval step using formic acid and trypsin. The primary antibody was incubated overnight at room temperature. The secondary antibody was incubated for one hour followed by a 30 minutes incubation with avidin-biotin complex (ABC, Vector Labs, CA, USA). Signal enhancement was completed by immersion in 3,3'-Diaminobenzidine (DAB). Finally, sections were counterstained with Harris Haematoxylin.

Adjacent sections were stained with the Von Kossa technique to detect calcification and a standard hematoxylin & eosin (H&E) staining. For the detection of iron, two protocols were used. For each case one section was stained using the classic Perls' Prussian blue iron stain. In addition, one section was stained according to an in-house protocol of a modified Meguro staining (9). In short, sections were incubated for 30 minutes in 1% potassium ferrocyanide, washed, followed by 60 minutes incubation in

methanol with 0.01 M NaN$_3$ and 0.3% H$_2$O$_2$. Subsequently, sections were washed with 0.1 M phosphate buffer followed by 30 minutes incubation in a solution containing 0.025% 3'3-DAB-tetrahydrochloride (DakoCytomation) and 0.005% H$_2$O$_2$ in 0.1 M phosphate buffer. The reaction was stopped by washing in demi water.

Tissue blocks from the sCAA case were processed in a similar fashion. Sections underwent histological staining with H&E, Perls' Prussian blue iron, and Von Kossa, and immunohistochemistry against Aβ (6F/3D, Dako).

All slides were digitized using an automatic bright field microscope (Philips Ultra Fast Scanner, Philips, Netherlands) for microscopic evaluation. All stained sections were independently evaluated by LGM, MB and SvV for the amount of Aβ, iron and calcium positive vessels. For both HCHWA-D subjects one area with the striped cortex, one area without the striped cortex and one area with some contrast changes were scored. Percentages were calculated for the amount of vessels positive and negative for iron and calcium with respect to the total number of Aβ positive vessels.

Results

Detection of the striped cortex *ex vivo*

Post mortem brain material of four HCHWA-D were scanned at 7T and in two out of four HCHWA-D cases a striped pattern was found *ex vivo* in the occipital cortex, similar to the *in vivo* MRI observation (Fig. 2A). In both HCHWA-D cases, also areas with normal contrast were observed on high-resolution *ex vivo* 7T MRI. In one out of six cases with sCAA screened for this study, a similar striped pattern as in the HCHWA-D cases was observed in the occipital cortex (Fig. 3A).

µCT and histopathological correlates of the striped cortex

Given the shape and course of the striped cortex, we hypothesised that the observed MRI contrast changes may be caused by Aβ depositions in the walls of perforating arteries, co-localizing with iron accumulation and calcifications.

Microscopic examination showed severe and widespread CAA in both HCHWA-D cases (Fig. 2C). The sCAA case was also characterised by severe and widespread CAA and more than 200 microbleeds on *ex vivo* 3T MRI (Fig. 3B). No noticeable differences in vessel wall morphology or Aβ staining of angiopathic arterioles was observed between areas with and without the striped cortex. Old microhemorrhages in the form of focal hemosiderin deposits were not evident in the examined tissue sections.
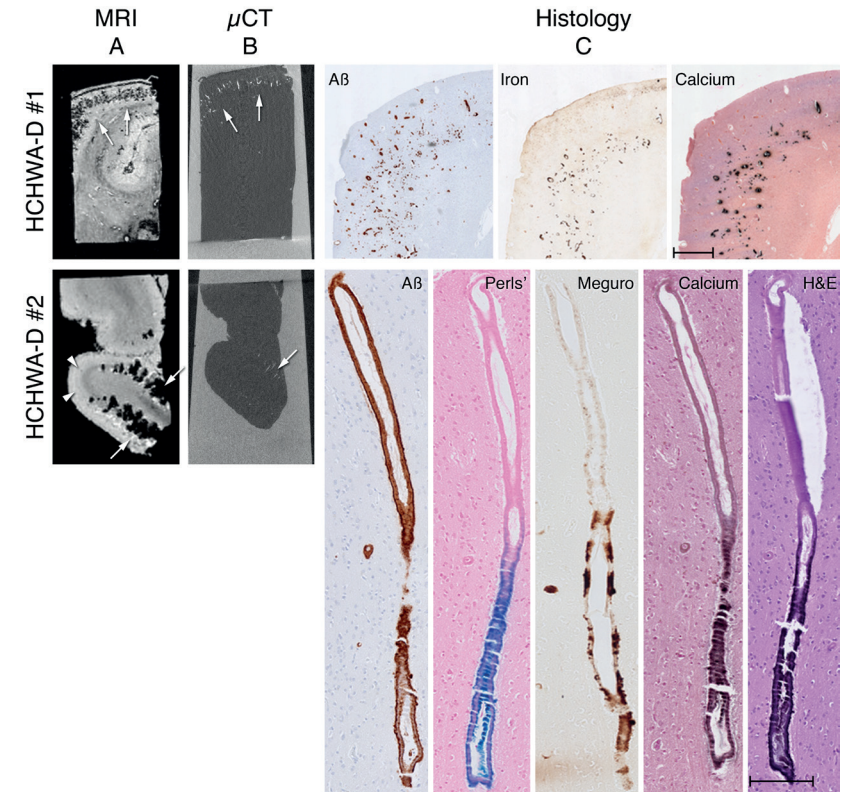
Figure 2: MRI, µCT and histology of the striped cortex in HCHWA-D. A striped pattern resembling the *in vivo* findings was found in the occipital lobe of HCHWA-D patient 1 (A, first row) and HCHWA-D patient 2 (A, second row). High-resolution 7T MRI (TE 12.5 ms) showed a striped pattern of the cortex (arrows) in addition to some parts of the cortical ribbon showing normal cortical contrast (arrowheads). (B) Vascular calcifications were confirmed by µCT (arrows). (C) Histopathological examination showed that the majority of the cortical vessels stained positive for Aβ. Interestingly, only vessels in the middle to deeper cortical layers stained positive for non-heme iron (Meguro) and calcium (Von Kossa), indicating iron accumulation and calcification of the vessel wall in specific cortical layers. In contrast to Aβ, which was observed over the full length of the perforating arteries, non-heme iron (Perls' and Meguro) and calcium (Von Kossa) was only observed in the lower part of the vessel. Calcification of the vessel wall was also visible on the H&E staining as deep purple depositions. C first row as well as C second row are consecutives slides. Orientation histology: top = leptomeninges; bottom = white matter. Scale bars in C first row = 1mm, C second row = 200 µm.
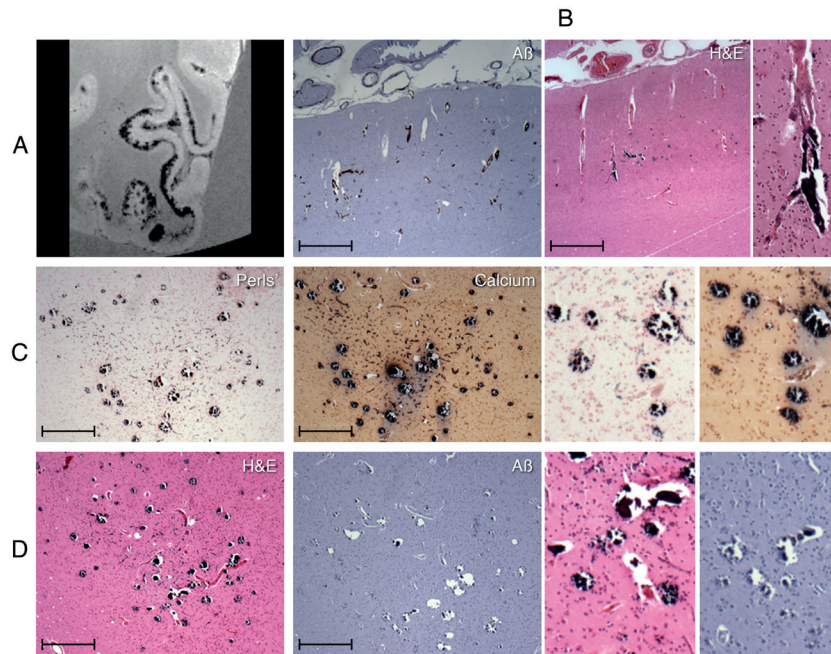
Figure 3: Striped cortex in a sporadic CAA case. (A) A similar striped pattern as in the HCHWA-D cases was observed in the occipital cortex of a sporadic CAA case on high-resolution 7T MRI. (B) This case was characterised by severe and widespread CAA and more than 200 microbleeds on ex vivo 3T MRI. (C) Histological examination showed the same co-localization in the vessel wall as observed in the HCHWA-D patients, namely: non-heme iron (Perls') and calcium (Von Kossa). Calcification of the vessel wall was also visible on the H&E staining as deep purple depositions. (D) Interestingly, in contrast with the HCHWA-D cases, iron accumulation and calcification was also observed in vessels without CAA. Scale bars = 500 μm.

In both HCHWA-D cases and the sCAA case, co-localization of Aβ with non-heme iron and calcium in the vessel wall was found in areas containing the striped cortex (Fig. 2, 3, Suppl. Fig. I, Suppl. Fig. II, Suppl. Table I). In contrast to Aβ, which was observed over the full length of the perforating arteries, non-heme iron and calcium were only observed in the lower part of the vessel (Fig. 2C). Calcification of the vessel wall was also visible in the H&E staining as deep purple depositions and confirmed by μCT (Fig. 2, 3). Interestingly, in contrast to the HCHWA-D cases, in sCAA, iron accumulation and calcification was also observed in vessels without CAA (Fig. 3D).

In addition, a hypointense line in the superficial cortical layers parallel to the cortical surface was observed in one of the HCHWA-D cases, resembling superficial siderosis. Histology showed co-localization with local iron accumulation in neurons in layer II of the cortex (Suppl. Fig. III).
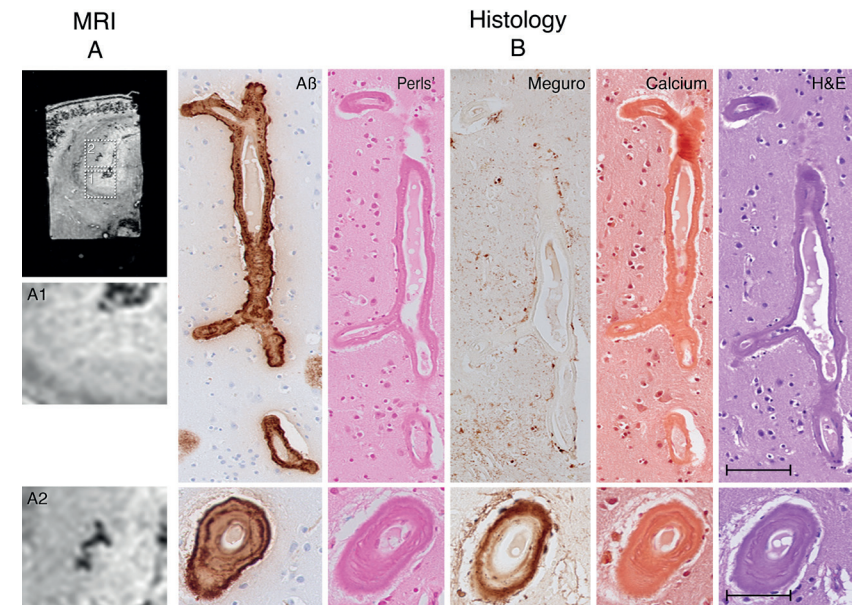
Figure 4: Zoomed MRI and histology of an area outside the 'striped cortex' in HCHWA-D patient 1. High-resolution 7T MRI (TE 12.5 ms) and zoom of an area without the striped cortex (A1) and an area with some contrast changes (A2). (B) Histopathological examination showed that most cortical vessels stained positive for Aβ in both areas. However, in areas without the striped cortex vessels were negative for non-heme iron (Perls' and Meguro) and calcium (Von Kossa). Also the H&E staining was negative. In areas with some contrast changes (A2), although less intense, these contrast changes correlated with an area where we found vessels positive for non-heme iron detected by the Meguro staining. The Perls', calcium and H&E stainings were negative. (B) are consecutives slides. Orientation histology: top = leptomeninges; bottom = white matter. Scale bars in B-F = 100 μm; G-K = 50 μm.

Histopathological correlate of areas outside the striped cortex

To confirm specificity of iron accumulation and calcification in the vessel wall as the histopathological correlate of the striped cortex, we further investigated other regions of the cortex in HCHWA-D tissue (Fig. 4A).

On histology, most cortical vessels stained positive for Aβ, also in areas without the striped pattern on MRI. However, these vessels were negative for non-heme iron and calcium (Fig. 4B, Suppl. Table I). We also noticed some areas outside the striped cortex with contrast changes on MRI which had a similar pattern, but that were less intense than the striped pattern described above. These MRI contrast changes may be due to vessels positive for Aβ and non-heme iron, but negative for calcium (Fig. 4B, Suppl. Table I). Calcification without iron accumulation was not observed.

Discussion

Our findings show that the recently observed striped pattern in the cortex of symptomatic HCHWA-D patients at 7T MRI is due to iron accumulation and calcification of Aβ positive penetrating arteries. The presence of both non-heme iron and calcification on penetrating arteries causes signal loss and hence abnormal cortical patterns on T_2^*-weighted MRI.

As reported, on *in vivo* 7T MRI the striped cortex was found in 40% of the symptomatic HCHWA-D patients. Since the striped cortex was not found in presymptomatic mutation carriers, it might be associated with more advanced stages of the disease (3). Typically, the first stroke occurs between the ages of 39 and 76 years (mean 50 years) and is fatal for one-third of the patients. Those who survive suffer from recurrent strokes resulting in severe disabilities. In exceptional cases, people survive longer, with a maximum reported survival of 28 years (10). Histopathological examination showed that both the HCHWA-D cases and the sCAA case were characterized by a severe and widespread CAA. This suggests that the striped cortex may be a marker of advanced CAA.

Mineral substances such as iron and calcium are known as paramagnetic materials and have consistently been reported to cause hypointense signals on T_2^*-weighted MRI images (5, 11). It is known that in patients with Alzheimer's disease and concomitant severe CAA have higher non-heme iron content in the temporal lobe than Alzheimer patients without CAA and that divalent iron accumulates within CAA-positive vessel walls (12). Iron-positive CAA vessels have also been reported in HCHWA-D patients (13, 14). Moreover, some of the largest cortical CAA vessels positive for iron were observed on histology and could be topographically matched with *ex vivo* high-resolution 9.4T MRI (13). Corresponding to our results, Nabuurs et al. showed that not all CAA-affected vessels resulted in a similar loss of MR signal (13). These different effects on MRI were thought to be caused by differences in the amount of Aβ, its positioning in the vessel wall or other associated microvasculopathies. Here we showed that Aβ depositions in and along the penetrating vessels are not in itself resulting in T_2^*-weighted MRI contrast; CAA vessels without iron accumulation and calcification resulted in normal MRI signal, whereas the presence of iron alone or both iron and calcium resulted in the detection of hypointense signals on T_2^*-weighted MRI.

These iron-sensitive MRI scans have been previously used to detect for example superficial siderosis, large intracerebral haemorrhage and (cortical) microbleeds. MRI-observed microbleeds, visible as small round hypointense lesions on T_2^*-weighted MRI, correlate to acute microhaemorrhages or iron-positive old microhaemorrhages on histopathology (6). Of note, in our cases the observed striped cortex was not associated with acute or old haemorrhages on histopathology.

Apart from iron accumulation in CAA vessels, we also observed calcification of the vessel wall. Calcification of CAA vessels has been previously reported in very severe CAA cases; cortical calcifications were found using histopathology in p.Glu692 Gln (Dutch) patients (15) and using CT in patients with p.Glu693Lys (Italian) and p.Asp694Asn (Iowa) mutations (16). However, iron accumulation and calcifications as detected by 7T MRI in patients with the p.Glu693Gln mutation (Dutch) have not been reported previously and the underlying mechanisms of vascular iron accumulation and calcifications in APP mutation carriers are unknown. The striped cortex was found in the occipital cortex on *in vivo* MRI (3), which seems to be consistent with the posterior predominance of CAA severity. In addition, we showed that predominantly vessels in the middle cortical layers accumulated iron and calcium. Why specific regions and cortical layers are more affected than others remains to be elucidated. Lastly, we showed that some vessels only accumulated iron but no calcification, whereas we did not find any vessels with calcifications without iron. Based on these observations we speculate that in HCHWA-D Aβ depositions in the vessel wall are followed by iron accumulation, resulting eventually in calcifications of the vessel wall.

Conclusions

In conclusion, we identified iron accumulation and calcification of the vessel wall in HCHWA-D as the histopathological correlates of the striped cortex observed on *in vivo* 7T MRI. This novel MRI marker is of interest for clinical CAA severity evaluation as it was also observed in a case with severe sCAA. Associated mechanisms resulting in iron accumulation and calcification of CAA vessels will need further investigation.

Disclosures
None

Supplementary Material
The Supplementary Material for this article can be found online at:
https://www.ahajournals.org/doi/suppl/10.1161/STROKEAHA.118.021872

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Chapter 5

Osteopontin and phospho-SMAD2/3 are associated with calcification of vessels in D-CAA, an hereditary cerebral amyloid angiopathy

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Abstract

In severe forms of cerebral amyloid angiopathy (CAA) pathology, vascular calcification has been observed in the cerebral cortex, both *in vivo* on MRI and CT, and *post mortem* using histopathology. However, the pathomechanisms leading to calcification of CAA-laden arteries are unknown. Therefore we investigated the correlation between calcification of cortical arterioles and several potential modulators of vascular calcification using immunohistochemistry in a unique collection of brain material of patients with a hereditary form of CAA, namely hereditary cerebral hemorrhage with amyloidosis-Dutch type (HCHWA-D or D-CAA).

We show a topographical association of osteopontin (OPN) and TGFβ signaling factor phospho-SMAD2/3 (pSMAD2/3) in calcified CAA vessel walls. OPN and pSMAD2/3 gradually accumulate in vessels prior to calcification. Moreover we found that the vascular accumulation of Collagen 1 (Col1), OPN and pSMAD2/3 immunomarkers correlated with the CAA severity. This was independently of the vessel size, including capillaries in the most severe cases. We propose that calcification of CAA vessels in the observed HCHWA-D cases may be induced by extracellular OPN trapped in the fibrotic Col1 vessel wall, independently of the presence of vascular amyloid.

Introduction

Hereditary cerebral hemorrhage with amyloidosis-Dutch type (HCHWA-D) is an autosomal dominant genetic disorder caused by a missense mutation on chromosome 21 in the amyloid precursor protein (APP, NP_000475.1:p. Glu693Gln, known as APP E693Q) (29). This so-called Dutch variant, or D-CAA, is characterized by early onset cerebral amyloid angiopathy (CAA) defined by a progressive accumulation of Amyloid beta (Aβ) in the cerebral vasculature, resulting in acellular thickening of the vessel wall (1), leading to recurrent hemorrhagic strokes with midlife onset (15, 18). CAA pathology is also present in the majority of Alzheimer's Disease (AD) patients and is associated with intracerebral hemorrhages in the elderly.

Recently, a striped pattern of hypointense lines perpendicular to the pial surface was observed on *in vivo* 7T MRI in the occipital cortex of symptomatic D-CAA patients (17). Histopathological examination showed that the striped pattern in the cortex on 7T MRI is due to iron accumulation and calcification of penetrating arterioles (5, 39).

Similar calcifications have been observed in other hereditary CAA variants, such as the Iowa and Italian variants (26, 33, 44). In sporadic CAA, calcification of cortical areas involving CAA-affected vessels seems to be much rarer than in the familial cases; its has only been described in three cases of CAA-associated cerebral hemorrhage (5, 20, 28). Intracranial vascular calcifications in general aging and in neurodegenerative diseases are frequently found in the basal ganglia (23, 41), but cortical calcification has not been reported. Therefore, calcifications of the CAA-vessels are thought to be one of the CAA-associated microvasculopathies, secondary to CAA (22, 39). However, it is unknown why some CAA-vessels exhibit calcifications, whereas other vessels with similar CAA load do not.

In the current study we investigated if vascular calcification in HCHWA-D is linked to expression of several factors known to promote calcification, independent of CAA itself, namely transforming growth factor β (TGFβ), osteopontin (OPN) and collagen1 (Col1), as detailed below. We previously reported an upregulation of the TGFβ pathway in HCHWA-D *post mortem* brain tissue (9, 10). In particular, we described in angiopathic vessels a correlation between CAA severity and the accumulation of phosphoSMAD2/3 (pSMAD2/3), a direct TGFβ1 downstream signaling factor (9). Since, it is known that TGFβ is a key factor in peripheral vascular calcifications (13, 40), we investigated the correlation between TGFβ regulation and calcification in individual vessels.

A second known modulator of vascular calcification is OPN, also known as secreted phosphoprotein 1 (SPP1). It is a non-collagenous bone matrix protein induced by TGFβ, leading to vessel calcification in a wide variety of peripheral disorders such as atherosclerosis, diabetes mellitus, and chronic renal failure (14). In the central nervous system, OPN is a constituent of the normal extracellular matrix (ECM), but in the context of neurodegenerative diseases, OPN has been described as a cytokine with a dual role in neuroinflammation and neuroprotection (4, 6, 43). Very little is known about the role of OPN in cerebrovascular calcification, though one previous study showed OPN associated with calcifications in the basal ganglia of AD patients (8).

Finally, ECM-related pathways are known to be upregulated in D-CAA (10). In particular collagen-1 (Col1), which is a pro-fibrotic gene induced by TGFβ1 signaling and an OPN binder, was found upregulated in D-CAA (9) and in an earlier study significantly increased quantities of Col1 were associated with vascular amyloid deposition (38). In atherosclerosis, Col1 is involved in vascular calcification through the binding of matrix vesicles, that act as nucleation foci for calcium deposits (11).

In order to unravel these pathomechanistic factors associated with cortical calcification, we used *post mortem* human brain material of D-CAA patients with well-characterized different levels of CAA load (9). We investigated the different layers (luminal, medial and abluminal) of calcified vessels with standard immunomarkers for these vascular components and assessed in cortical area the association of pSMAD2/3, Col1 and OPN with calcification and disease severity.

Material & Methods

Brain tissue

Occipital *post mortem* brain tissue from D-CAA patients was obtained from the Netherlands Brain Bank (NBB) and from our local neuropathology tissue collection (LUMC). Written informed consent was obtained for each donor and all material and data were handled in a coded fashion maintaining patient anonymity according to Dutch national ethical guidelines (Code for Proper Secondary Use of Human Tissue, Dutch Federation of Medical Scientific Societies, Rotterdam, the Netherlands). The study was approved by the local Ethics Committee.

Experimental design

Brain tissue from eight D-CAA patients, genetically tested for the presence of the mutation (NM_000484.3(APP):c.2077G>C), was used in this study as summarized in Table 1. In addition to the two cases previously described for the presence of calcifications (in (5); cases H2 and H14), six additional patients presenting a known range of CAA severity (based on (9)) were selected (H5, H6, H7, H8, H9 and H10 as shown in Table 1).

Table 1: Demographics of cases, patient code and CAA severity based on (9)

Diagnosis	Source	Age	Gender	PMDa	Codeb	CAA severity c
HCHWA-D	LUMC	70	F	5	H14*	n.a.
HCHWA-D	LUMC	57	M	3	H2*	+
HCHWA-D	LUMC	50	M	19	H5	+
HCHWA-D	LUMC	51	M	3	H8	+
HCHWA-D	NBB	61	M	7	H6	++
HCHWA-D	LUMC	71	M	n.a.	H10	++
HCHWA-D	LUMC	67	F	n.a.	H9	+++
HCHWA-D	NBB	71	M	6	H7	+++

Abbreviations: HCHWA-D = hereditary cerebral hemorrhage with amyloidosis-Dutch type, NBB = Netherlands Brain Bank, LUMC = Leiden University Medical Center, n.a.= not available.

a *Post mortem* delay (in hours)
b Patient code used in (9), (added material in italic)
c +: moderate, ++: high, +++: very high
* Patient material used in studies described in (5)

Tissue sectioning and staining

Formalin-fixed, paraffin-embedded blocks of brain tissue were cut into serial 5 μm thick sections and mounted on coated glass slides (SuperFrost® Plus, VWR, Dietikon, Switzerland). Deparaffinization in xylene and rehydration through a series of ethanol concentrations was done. To detect calcification standard hematoxylin & eosin (HE) staining and the Von Kossa technique were included. For immunohistochemistry, the usage of an antigen retrieval procedure, a list of antibodies and their dilution are mentioned in Supporting information Table S1. After blocking steps for endogenous peroxidase (3 % H_2O_2 in dH_2O for 10 minutes) and for unspecific epitopes binding in blocking buffer (1 % BSA suspension in 0.1 % Tween 20, Phosphate Buffer Saline pH 7.4 for 1 hour at room temperature), the sections were incubated with primary antibody overnight at 4°C in the blocking buffer. Incubation with secondary HRP antibody was

followed by a DAB reaction (standard or enhanced using an avidin-biotin complex; details in Supporting information Table S1) and mounted with Entellan® New (107961, Merckc, Darmstadt, Germany). An overview of the serial sectioning with the sequence of stainings is given in Supporting information Table S2. All slides were digitized using an automatic bright field microscope (Philips Ultra Fast Scanner 1.6 RA, Philips, Netherlands). All stained sections were independently evaluated by LvdG and LGM for the amount of different immunomarkers as described below.

Quantification of severity grade of calcification, Col1 load, CAA load (Aβ), osteopontin load and pSMAD2/3 load

On the HE stained sections, three cortical areas were delimited at low magnification (2.016 mm^2 per field of view), based on the presence of calcifications (deep purple color; (24)). The corresponding area on consecutive slides immunostained for OPN, pSMAD2/3, Aβ and Col1 was identified for each patient. Every area was counted twice at higher magnification (6.897 mm^2 per field view): one time for the total number of stained vessels and one time for the stained capillaries only. Subtraction of capillary number to total number of vessels gave the amount of intermediary size vessels. This group include all vessels larger than capillaries, with a typical external diameter between 10 and 300um. Leptomeningeal vessels were excluded. Of note, only fully calcified vessels with a deep purple color in HE were counted.

Statistical analysis was done using GraphPad Prism v7.00 with a level of statistical significance set at p <0.05. Normality of the distribution was assessed with a D'Agostino & Pearson test. Correlation matrices were computed with a two-tailed p-value using Pearson correlation or with Spearman correlation in case of non-parametric distribution.

Quantification of immunomarkers in calcified vessel:

Classic vascular immunomarkers were investigated in the calcified vessel layers (luminal, medial and abluminal). Vascular smooth muscle cells (VSMCs) are labeled with Smooth Muscle Actin (SMA) and the endothelial cells (ECs) with CD31, CD105, vWF and/or ICAM-1. The collagen composition of the ECM was determined by Col4 and Col1.

Intact calcified vessels that could be identified on consecutive sections were scored for SMA, CD31, ICAM-1, CD105, vWF, Col4 and Col1 in the luminal, medial and abluminal layers (examples are given in Supporting information Figure S1). A maximum of 10 vessels per patient was graded, given a total of 48 vessels scored in the different patients: 0 vessel for H5; 3 vessels for H8; 10 vessels for H10; 4 vessels for H2; 3 vessels for H9; 10 vessels for H6, 8 vessels for H14, and 10 vessels for H7.

Results

Calcified vessels and localization of immunomarkers

We expect the majority of stained vessels to be small arteries and arterioles (primarily affected in this disorder), but a specific distinction with veins and venules was not assessed; therefore we use the term of ''medium to large vessels''. Furthermore, we will employ the term calcification, more widely used than mineralization, with the understanding that the deposits may consist apart from calcium of several other co-aggregating minerals such as iron.

Calcification severity and occurrence of OPN and pSMAD2/3 labeling

Calcifications of angiopathic vessels were found in the occipital cortex of 7 out of 8 D-CAA cases. We observed different calcified vessel load between the D-CAA cases, expressed as the number of fully calcified vessels per mm^2, as shown in Figure 1. Within individuals, calcified vessels were clustering in specific part of the tissue section, and confined to the middle cortical layers (as also described in (5)). Calcified capillaries were Aβ-laden capillaries (capCAA), restricted to areas where bigger calcified vessels were present (data not shown) and only observed in those cases with the most severe calcification load in the medium to large vessels (Figure 1).

Calcified vessels showed a topographical co-localization with OPN and pSMAD2/3 staining in consecutive slides (Figure 2). Calcified vessels were always positive for both OPN and pSMAD2/3. Longitudinal sectioning of individual penetrating CAA-laden arterioles showed amyloid deposits along the entire vessel wall, but an absence of calcification along the leptomeninges
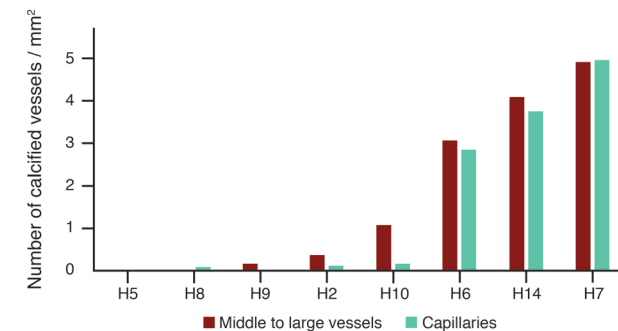
Figure 1: Calcification load (number of fully calcified vessels/mm2) in selected HCHWA-D material ranked from left to right on increased calcified vessel load. Calcification load range from: absent (in H5); low (in H8); mild (in H9, H2 and H10) to heavy (in H6, H14 and H7).

and the superficial cortical layers. Mid-way through the cortex, the gradual transition from non-calcified to calcified vessel wall was accompanied by the occurrence of OPN and pSMAD2/3 granules showing the same gradual transition (Figure 3). Within individual patients, CAA-laden vessels with different calcification load could be identified within one cortical region (Figure 4). Although calcified vessels were always OPN and pSMAD2/3 positive, the reverse is not the case: some OPN and pSMAD2/3 staining was detected in non-calcified angiopathic vessels as well.
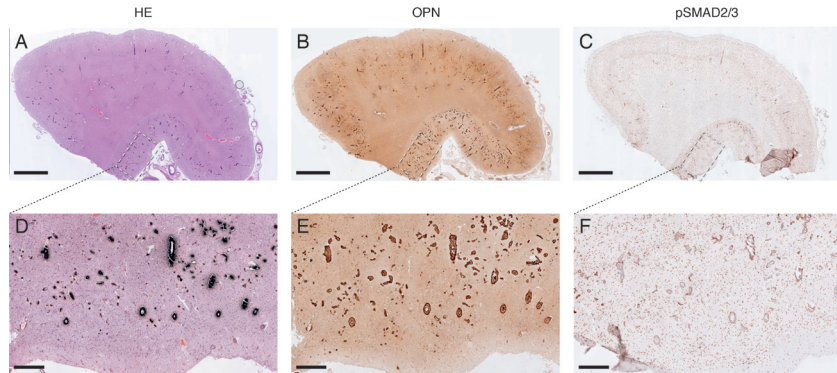
Figure 2: Topographical co-localization of OPN and pSMAD2/3 staining in calcified vessel already detectable at low and medium magnification; H7 patient. A, D. von Kossa staining. B, E. OPN staining. C, F. pSMAD2/3 staining. (A-C) Overview, scale bar 2mm; (D-F) Details of respectively (A-C); scale bar 400μm.
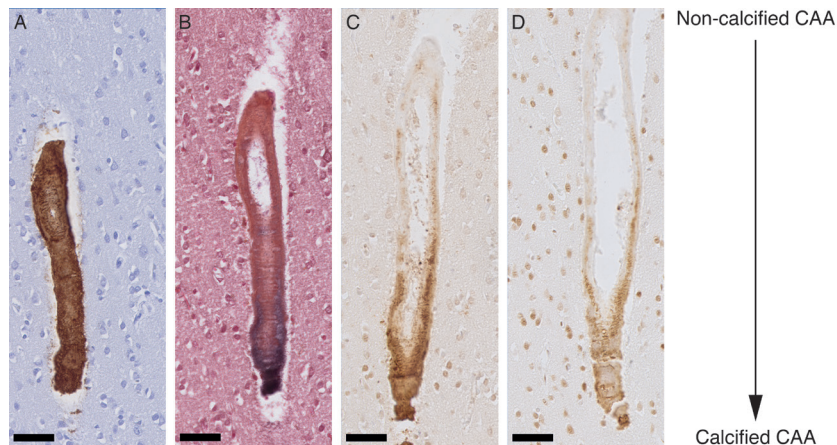
Figure 3: Transition CAA non-calcified to calcified vessel showing the gradual medial OPN and pSMAD2/3 accumulation. Longitudinal sectioning of a penetrating artery oriented leptomeninges (top) to white matter (bottom) side; H2 patient. A. Aβ staining. B. von Kossa staining. C. OPN staining. D. pSMAD2/3 staining. A-D. Consecutive slides; scale bar 50μm.

Vessels	I	II	III	IV
Aβ	+	+	+	+
Calcification	-	-	+	+
OPN	-	+	+	+
pSMAD2/3	-	+	+	+
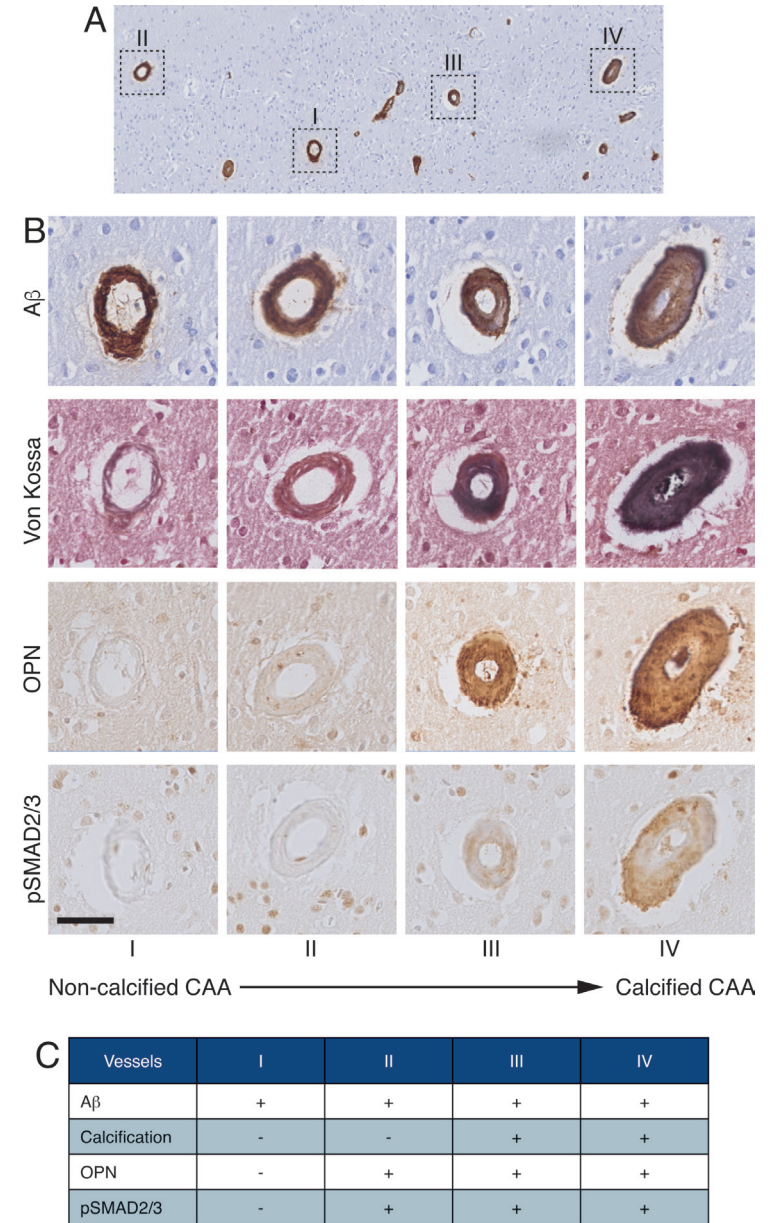
Figure 4: Adjacent cortical angiopathic vessels showing different degree of calcification and gradual accumulation of OPN and pSMAD2/3 in transversal sectioning, H2 patient. A. cortical area overview stained for Aβ; scale bar 200μm. B. details of vessels I to IV identified in (A) and consecutive slides labeling, scale bar 50μm. C. Table indicating the presence (+) or absence (-) of labelling on the vessel wall.

OPN also labeled the nuclei and cytoplasm of some glial cells and neurons. Notably, perivascular cells with a strong cytoplasmic OPN staining were detected in the proximity of vessels undergoing calcification (Supporting information Figure S2). In the parenchyma, pSMAD2/3 labeling was predominantly located in the nuclei of neurons, with little or no cytoplasmic staining, as previously described (9).

Characterization of the layers in the calcified vessels

Studies in peripheral vessels show that diverse mechanisms may trigger calcification, with different vascular components or cell types involved, such as the vascular smooth muscle cells (VSMCs), the endothelium or the extracellular matrix. Therefore we investigated the different layers (luminal, medial and abluminal) of the calcified vessels in more detail.

In D-CAA, the VSMCs have degenerated, as shown by the absence of SMA in the medial layer (Figure 5A). However, the endothelial layer is thought to remain intact (31). Nevertheless, a recent study assessing endothelial dysfunction in CAA showed a loss of CD31 in AD with CAA vessels compared to AD without CAA (21). As endothelial dysfunction may trigger inflammation and is closely linked with vascular pathology, including peripheral vascular calcification, we investigated normal (CD31, CD105, vWF) and activated (ICAM-1) endothelial markers.

Lumen were most frequently labeled for vWF (73% of calcified CAA vessels; Figure 5B). Classical endothelial labeling CD105 and CD31 remained positive in only 35% and 29% of the calcified CAA vessels, whereas luminal SMA staining was detected in 8% of the quantified vessels. An absence of luminal immunostaining was scored in 4% of the calcified vessels. The endothelial inflammation immunomarker ICAM-1 was present in 21% of the calcified lumens whereas ICAM-1 on astrocyte end foot was the major abluminal staining (96% of the scored calcified vessels; Figure 5B).

ECM remodeling of the basement membrane is known to occur in CAA, initially at the abluminal side. As Col1 is a known modulator of calcification, we investigated the presence of normal (Col 4) and fibrotic (Col 1) collagen. Col1 staining was more frequent than Col4 staining in abluminal (71% vs 10%) and medial layers (25% vs 2%) but not in luminal layers (58% vs 65%; Figure 5B).

In accordance with endothelial dysfunction and ECM remodeling, calcified vessels presented a reduction in classical luminal immunomarkers and an intensification in medial and abluminal fibrotic Col1. The above changes in the different layers were also observed in non-calcified CAA vessels but much less frequent.
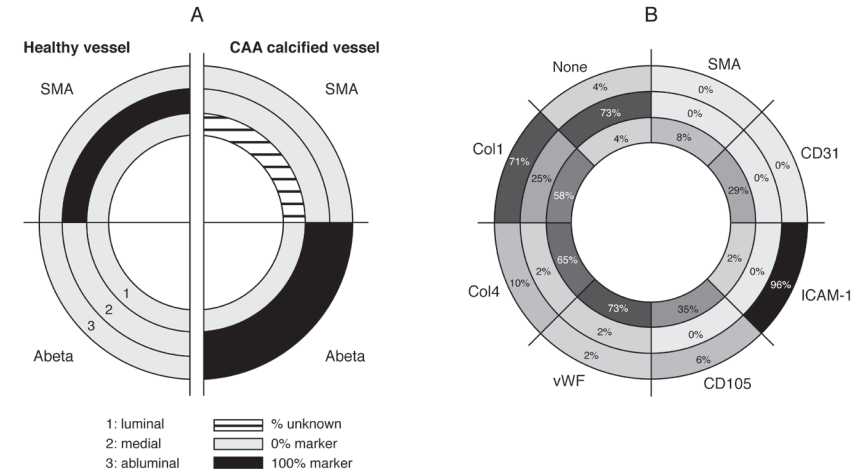
Figure 5: Characterization of calcified vessels. A. Representation of Aβ and SMA labeling in the luminal, medial and abluminal vascular layers in healthy and calcified CAA vessels in HCHWA-D. Luminal= endothelium; medial = VSMCs; abluminal = external basement membrane and astrocytic end feet. B. Representation of the proportion of each staining occurrence (or absence of) per vessel layers. Most of the vessel layers stained for multiple markers.

Vascular accumulation of Col1, OPN and pSMAD2/3 immunomarkers is related to disease severity

We observed that the majority of CAA vessels and capCAA (Aβ-laden capillaries) in D-CAA were Col1 positive; anatomically normal vessels were Col1 negative. An illustration of the topographical association between Col1 and Aβ in the angiopathic vessel wall is presented in Supporting information Figure S3 for two cases with different CAA and capCAA severity . CAA and CapCAA vessels were mostly OPN positive, but also frequently pSMAD2/3 positive.

Apart from a topographical association, we also investigated the accumulation of OPN, pSMAD2/3 and Col1 in the vessel wall in relation to disease severity as measured by CAA or capCAA load. Positive significant correlations between all immunomarkers studied and disease severity were found (Figure 6 and Supporting information Figure S4). Independently of the vessel size, the correlation of CAA/capCAA load with OPN, pSMAD2/3 and Col1 load was most significant.
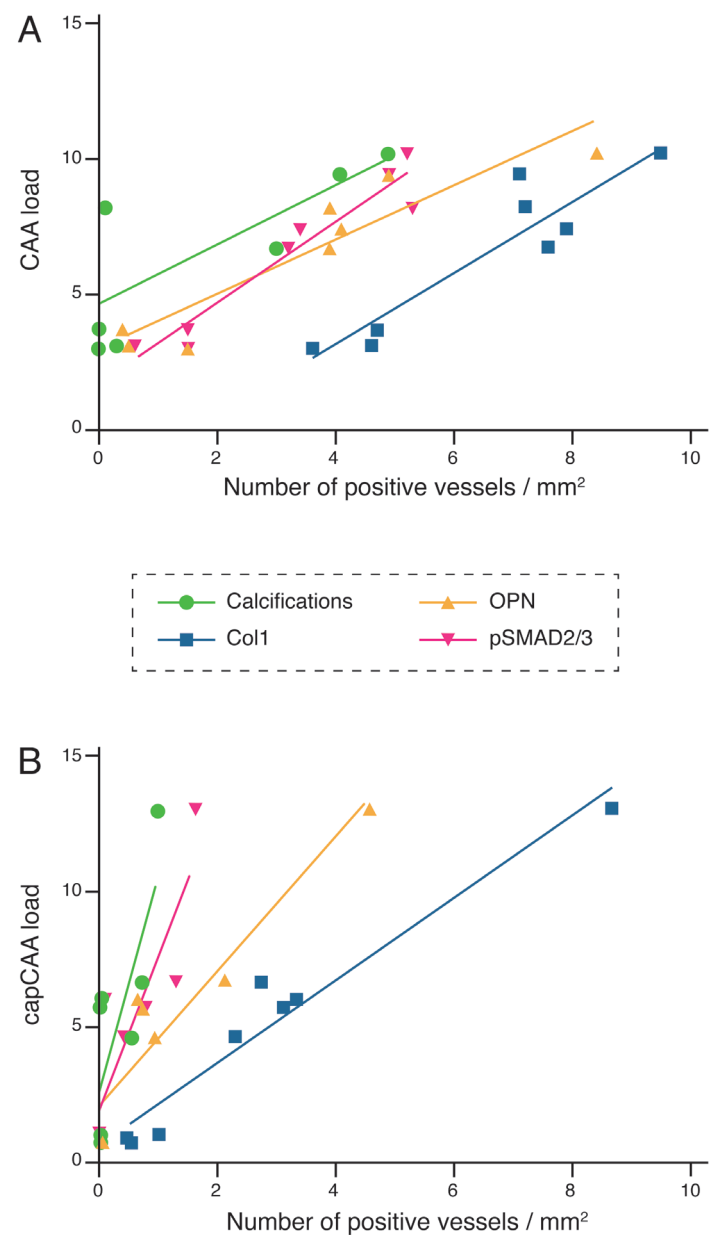
Figure 6: Graphical representation of the quantified OPN, pSMAD2/3 and Col1 immunomarkers and calcified vessels per patient plotted against the CAA load. A) in medium to large vessels and B) in capillaries. Highest correlation coefficient was obtained in A) between the CAA load and the pSMAD2/3 or OPN load (respectively Pearson r=0.96, p=0.0001 and r=0.93, p= 0.0009). Details are given in Supporting information Figure S4.

Discussion

This study investigated OPN and Col1 immunomarkers though to promote calcification together with Aβ and pSMAD2/3 known immunomarkers associated with CAA severity, and proposes a mechanism of vascular calcification in HCHWA-D.

Calcifications are a feature of late stage CAA and capCAA pathology

In this study we found a significant correlation of the occurrence of vascular calcification with CAA severity, in line with a previous study on CAA-associated microvasculopathies (including calcifications) which also showed a correlation with CAA load (27).

Despite the spatial correlation between CAA load and vascular calcification, to date there is no proof for causality between the presence of the Aβ peptide per se, and calcification of the vessel wall (10,13). This is further supported by our previous finding where we observed calcification of non-CAA vessels in a sCAA case (6). Furthermore, we observed in the present and in previous studies very heterogeneous patterns of calcification in areas with comparable CAA load, even within the same patient. This suggests that vascular calcifications are most likely a secondary complication of the CAA pathology, but not directly caused by the deposition of amyloid.

As described in prior studies, capCAA is detected only in severely affected HCHWA-D patients and increases with CAA load (27,28). Calcifications of capCAA occurred mostly in subjects with the highest calcified vessel load and in proximity to other calcified vessels. This might either indicate a spreading of the calcification over the entire vascular tree, or it could be the result from surrounding parenchymal changes. Given the preferential occurrence of calcification in specific cortical lamina (6), local changes in the surrounding parenchyma are more plausible.

Col1, OPN and pSMAD2/3 accumulation might precede vessel wall calcification

To investigate the pathomechanism of vascular calcification in CAA, we investigated several potential modulators. pSMAD2/3 is a direct TGFβ1 downstream signaling factor which is known to modulate peripheral vascular calcification. OPN is a matrix protein induced by TGFβ, also leading to peripheral vessel calcification and a central nervous system constituent of the ECM. Lastly, we stained for Col1, a pro-fibrotic gene induced by TGFβ1 signaling and an OPN binder, involved in ECM remodeling.

In Figure 6, the order of progression for each immunomarker is visualized as a function of increasing CAA severity. Interestingly, for the same scored area the number of vessels labeled by Col1 was often higher than the number of vessels stained for Aβ (Supporting information Figure S4B), indicating that the fibrosis of the vessel wall might precede the amyloid deposition. Alternatively, as the arterial system was not distinguished from the venous system, the fibrosis of veins and venules (usually spared from amyloid deposition) might contribute to the number of Col1-positive vessels as well. Both scenarios involve an early stage fibrosis of the vessel wall due to ECM remodeling regulated by TGFβ as proposed in our previous work (16,17).

In addition, the topographical co-localization and gradual accumulation of OPN and pSMAD2/3 with calcification reveals that OPN and pSMAD2/3 accumulation are tightly linked to the calcification process. Moreover, the presence of OPN and pSMAD2/3 accumulation in non-calcified angiopathic vessels suggests an ongoing calcification process.

TGFβ and OPN changes in calcified vessels

Besides having a role in excessive vascular fibrosis, prolonged chronic induction of TGFβ might directly contribute to the calcification process in HCHWA-D. Evidence of TGFβ-related induction of calcification genes were described in neuron-specific TGFβ1-overexpressing transgenic mice that presented with cerebral angiopathy. A microarray analysis of genes in the cortex identified upregulation of a group of genes involved in calcium homeostasis, tissue calcification and vascular calcification, including OPN (29).

In our studies, there was a topographical co-accumulation of OPN and pSMAD2/3 in pre-calcified vessels. This likely reflects a role for SMAD signaling in the vascular calcification. This is supported by Shi et al. who demonstrated that pSMAD3 can bind directly to the OPN promoter, activating OPN transcription (30). Furthermore SMAD3 overexpression in a mouse osteoblastic cell line was found to promote calcification via Col1 upregulation (31).

Calcification hypothesis in HCHWA-D

Many mechanisms have been proposed for vascular calcification in peripheral diseases. Cortical calcification in these paper resembles concentric medial artery calcification described in diabetes and chronic renal disease (32) and are different from intimal calcification as seen in atherosclerosis (eccentric; lumen deforming; (33)). In medial calcification, the proposed mechanisms are usually VSMCs mediated with vascular OPN production (34).

However, in this study, VSMCs are likely not responsible for the medial calcification. Firstly, VSMCs are not present in calcified, nor in pre-calcified OPN-positive vessels ((6) and this study) because CAA positive vessels usually do not have any VSMCs remaining. Furthermore, many CAA positive vessels were OPN-negative, suggesting that OPN and Aβ accumulation are caused by different mechanisms and vascular OPN does not have a vascular SMC origin.

This is based on our observation that there was local heterogeneity of vascular calcification, even within the same patient. We hypothesize that this variability may be due to tissue responses to local events such as microbleeds, micro-infarcts and hypoxia that are likely to occur in these patients. In this context, OPN is known to be upregulated and neuroprotective after ischemic events such as stroke (35) and local cortical injuries induce OPN increase in parenchymal cells as demonstrated in animal models of blood brain barrier damage in the context of ischemic (36) or hemorrhagic injuries (37). Furthermore, OPN is an immunomodulatory cytokine, upregulated in reaction to toxic amyloid species (38). Testing these hypotheses should be done in future studies using animal or cell models.

Lastly, it was demonstrated *in vitro*, that OPN could induce the calcification of Col1 fibers in the context of bone formation (39). In the current study the co-localization of OPN and Col1 in calcified vessels suggests a functional link between vascular OPN likely trapped into Col1 fibers and calcification of CAA vessel wall.

The clinical detection of potential biomarkers of vascular remodeling such as Col1 and OPN in blood or CSF, might give insight into the progression of CAA pathology in HCHWA-D. In addition, further investigations are required to determine whether OPN is a regulator of the early disease mechanism of interest as therapeutic target.

Acknowledgments

Compliance with ethical standards

Conflicts of interest: The authors declare they have no conflicts of interest.

Ethical approval: All procedures performed in this study involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent: Informed consent was obtained from all individual participants included in the study.

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Chapter 6

Brain transcriptomic analysis of hereditary cerebral hemorrhage with amyloidosis–Dutch type

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Abstract

Hereditary cerebral hemorrhage with amyloidosis–Dutch type (HCHWA-D) is an early onset hereditary form of Cerebral Amyloid Angiopathy (CAA) caused by a point mutation resulting in an amino acid change (NP_000475.1:p.Glu693Gln) in the Amyloid Precursor Protein (APP). *Post mortem* frontal and occipital cortical brain tissue from 9 patients and 9 age-related controls was used for RNA sequencing to identify biological pathways affected in HCHWA-D. Although previous studies indicated that pathology is more severe in the occipital lobe in HCHWA-D compared to the frontal lobe, the current study showed similar changes in gene expression in frontal and occipital cortex and the two brain regions were pooled for further analysis. Significantly altered pathways were analyzed using Gene Set Enrichment Analysis (GSEA) on 2036 significantly differentially expressed genes. Main pathways overrepresented by down-regulated genes were related to cellular aerobic respiration (including ATP synthesis and carbon metabolism) indicating a mitochondrial dysfunction. Principal up-regulated pathways were Extra Cellular Matrix (ECM)-receptor interaction and ECM proteoglycans in relation with an increase in the Transforming Growth Factor beta (TGFβ) signaling pathway. Comparison with the publicly available dataset from pre-symptomatic APP-E693Q transgenic mice identified overlap for the ECM-receptor interaction pathway, indicating that ECM modification is an early disease specific pathomechanism.

Introduction

Cerebral amyloid angiopathy (CAA) refers to the presence of amyloid, commonly Amyloid β (Aβ), in intracerebral vessels. CAA pathology is present in the majority of Alzheimer's Disease (AD) brains and is associated with intracerebral hemorrhages in the elderly.

Hereditary cerebral hemorrhage with amyloidosis-Dutch type (HCHWA-D) is a severe monogenic form of CAA with an autosomal dominant pattern of inheritance. A point mutation at codon 693 of the Amyloid Precursor protein (APP) located at chromosome 21 results in a glutamine for glutamic acid substitution (NP_000475.1:p.Glu693Gln) leading to the formation of the Aβ-E22Q peptide, a toxic variant of the Aβ peptide well-studied *in vitro* (1). Pathologically, HCHWA-D is characterized by severe amyloid angiopathy of meningo-cortical blood vessels; mutation carriers suffer from intracerebral hemorrhage, starting typically between the ages of 40 and 65.

Although previous radiological and neuropathological studies describe the disease in detail, the exact molecular processes causing Aβ accumulation in the vessel wall largely remain to be characterized. Some mechanisms have been already examined in HCHWA-D *post mortem* material, in particular extracellular matrix (ECM) remodeling in the angiopathic vessel wall. Changes in the basement membrane composition with accumulation of heparan sulfate proteoglycans (2) and activity of ECM-cross-linking enzymes like lysyl-oxidase (3) or tissue-transglutaminase (4) are processes known to promote Aβ aggregation. More recently, our group showed a phospho-Smad 2/3 dependent dysregulation in the TGFβ pathway (5) with an increase in pro-fibrotic transcripts.

Genome wide gene expression studies are an unbiased approach to attain a comprehensive picture of dysregulated genes and pathways. RNA sequencing of *post mortem* human brain tissue is of particular interest to unravel complex mechanisms in neurodegenerative diseases (6), such as AD (7–9). In the current study, we perform a pathway analysis on differentially expressed protein-coding genes in brain tissue of HCHWA-D patients and age-matched controls to detect potential novel therapeutic targets and biomarkers of CAA pathology and to better comprehend the molecular pathology of HCHWA-D.

Recently, the brain transcriptome of the transgenic APP-E693Q mice was published (10), which allowed us to study overlapping pathways between our HCHWA-D patient data and pre-symptomatic APP-E693Q transgenic mice, in order to identify conserved and critical pathways in CAA.

Patients, materials and methods

Study design

This study was performed in 36 samples: frontal and occipital cortex samples from 9 HCHWA-D and 9 non-demented control (NDC) subjects. Patient material information can be found in Figure 1. Both frontal and occipital cortex were used, based on the previous finding that the CAA pathology is more severe in the occipital lobe in HCHWA-D compared to the frontal lobe (11). Written informed consent was obtained for each donor in accordance with the Declaration of Helsinki and all material and data were handled in a coded fashion maintaining patient anonymity according to Dutch national ethical guidelines (Code for Proper Secondary Use of Human Tissue, Dutch Federation of Medical Scientific Societies). The study was approved by the local Ethics Committee (Commissie Medische Ethiek, LUMC).

RNA isolation, library preparation and sequencing

Brain tissue processing, tissue homogenization and RNA extraction were done as described previously (5) (see Datasheet 1_Supplementary Method). Samples for RNA extraction were processed directly, whereas samples for protein extraction were stored at -80C prior to analysis. RNA content was measured with the Nanodrop at 260nm and evaluation of RNA Integrity was performed with on-chip electrophoresis using an RNA 6000 Nano kit and a Bio-Analyzer 2100 (Agilent). Samples with a RNA integrity number (RIN) value of <5 were excluded from the study.

RNA samples were depleted for ribosomal RNA (rRNA) with the Ribo Zero Gold Human kit (Illumina) and strand specific RNA-Seq libraries were generated as previously described (12), with minor modifications as defined in Datasheet 1_Supplementary Method. After amplification of the libraries, samples with unique sample indexes were pooled and sequenced paired-end 2x50bp on a HiSeq2500 system following standard Illumina guidelines.

Mapping reads, gene expression counts and quality controls

RNA-Seq files were processed using the BIOPET Gentrap pipeline v0.6 developed at the LUMC[1] which performs FASTQ preprocessing (including quality control, quality trimming and adapter clipping), RNA-Seq alignment, read and base quantification, and optionally transcript assembly. FastQC (RRID:SCR_000141, v0.11.2) was used for checking raw read QC. Low quality read trimming was done using Sickle (RRID:SCR_006800, v1.33) with default settings. Adapter clipping was performed using Cutadapt (RRID:SCR_011841, v1.9.1) with default settings. RNA-Seq reads

1   http://biopet-docs.readthedocs.io/en/latest/releasenotes/release_notes_0.6.0/

alignment were performed using GSNAP (RRID:SCR_005483, v2014-12-23) with setting "--npaths 1" on GRCh38 reference genome without the alternative contigs. The gene read quantification was performed using HTSeq-count (RRID:SCR_011867, v0.6.1p1) with setting "--stranded reverse". The gene annotation used for quantification were UCSC RefSeq genes for GRCh38 downloaded on 2015-07-13.

SNPs and gender based quality controls as well as median 5'-3' bias methods are specified in Datasheet 1_Supplementary Method.

Normalization and differential expression analysis

CQN (RRID:SCR_001786, v1.22) was used to normalize the gene count table for library sizes, gene transcript lengths (sum of exons lengths) and gene transcript GC-contents (concatenated exon sequences). EdgeR (RRID:SCR_012802, v3.18.1) was used to perform differential gene expression analysis. An interaction model *group*area* was fit with two factors: *group* (two categories: HCHWA-D *vs*. control) and *area* (two categories: frontal *vs*. occipital). As offsets we used the normalization coefficients obtained from the CQN model. Benjamini & Hochberg FDR was computed to adjust *p*-values obtained for each differentially expressed gene (DEG). Finally, a table of raw and normalized expression levels of the genes was produced (not shown), and a table of gene expression levels fold changes (FC) and their significances for each gene and model component.

Validation of RNA-Seq data

Quantitative RT-PCR (qPCR) was performed with the same RNA extracts as were used for RNA-Seq analysis and the primers listed in Datasheet 2_Supplementary Table 1. Primer design, qPCR method and analysis were done as described previously (5) are provided in Datasheet 1_Supplementary Method. Correlation of expression levels between the RNA-Seq and the qPCR data was calculated from expression levels of selected genes extracted from the count matrix. The gene expression mean values per patient (log2 transformed) of both the count matrix and the qPCR results were plotted.

Western Blot was performed from frozen brain sections as described in Datasheet 1_Supplementary Method with anti-HSP70 (1:1500, Santa Cruz Biotechnology Cat# sc-24, RRID:AB_627760) and anti-β-actin (1:5000, Abcam Cat# ab6276, RRID:AB_2223210).

All statistical analysis from the validation section were conducted in GraphPad Prism (RRID:SCR_002798, v7.00) with a level of statistical significance set at $p < 0.05$.

Pathway analysis

Gene Ontology (GO) annotations and significantly altered pathways in KEGG, Reactome and Wikipathways were analyzed using GeneTrail2 (13) (RRID:SCR_006250, v1.5; GO, KEGG, Reactome retrieved 11/01/2016; Wikipathways retrieved 18/02/2016) with the Gene Set Enrichment Analysis (GSEA) method. GSEA was performed using a Kolmogorov-Smirnov non-parametric rank statistic (14) with Benjamini and Yekutieli FDR multiple testing adjustment method. Gene lists were ranked based on either FC or FDR (top list with most significantly up-regulated and bottom list with most significantly down-regulated; keeping the dysregulation direction).

Analysis was performed on extended gene sets and most significant DEGs from HCHWA-D brains from the current study and from transgenic APP-E693Q mice (DEG WT vs. APP-E693Q mice, entorhinal cortex (10) as depicted in Figure 2. Subsequent known interactions between genes set were visualized with the STRING database (RRID:SCR_005223, v10.5).

Results

Quality checks of samples and RNA-Seq reads

Based on SNPs, gender and HCHWA-D mutation presence, all 36 samples were concordant and included in the analysis (Datasheet 3_Supplementary Figure 1). The average number of reads after sequencing was 19 578 485 for controls and 23 038 641 for HCHWA-D. The number of reads after alignment are depicted in Datasheet 3_Supplementary Figure 2. The count matrix was generated with uniquely mapped reads. On average, the HCHWA-D samples had a lower RIN value than control samples (6.50 ±0.96 and 7.92 ±0.78 respectively). The median 5'-3' bias (ratio of median 5'bias:3'bias) was calculated for each sample (samples code and details in Datasheet 2_Supplementary Table 2). High values (>5) were found for 3 control samples (S_7, S_17, S_18) and 8 HCHWA-D samples (S_19, S_21, S_22, S_23, S_24, S_28, S_32, S_36), but we did not find a significant correlation with a longer *post mortem* delay (PMD) or a lower RIN value (not shown). On another hand, there was a strong positive correlation between the median 5'-3' bias and the number of reads with GC-content > 75% (Datasheet 3_Supplementary Figure 3). This prompted us to apply a GC correction before analyzing differentially expressed genes.

Quality controls of differential gene expression and analysis

Because of large differences in the GC-content of samples, correction for GC bias was done with the CQN model. After GC correction, fold changes dependence of gene GC-contents were no longer observed (Figure 3). The

clustering of reads was examined with principal component analysis (PCA) and no clustering related to the gender or the origin of tissue (NBB *vs*. LUMC; data not shown) were identified. PCA showed a close clustering of the control samples with less homogeneity in the HCHWA-D samples. Most frontal and occipital samples from the same subject clustered together (Datasheet 3_Supplementary Figure 4).

Diagnosis	Source	Age range	PMDs[a]
NDC	LUMC	70-75	6
NDC	LUMC	65-69	4
NDC	NBB	60-64	6
NDC	NBB	60-64	10
NDC	NBB	55-60	8
NDC	NBB	55-60	9
NDC	NBB	55-60	8
NDC	NBB	50-54	8
NDC	LUMC	40-45	n.a.
HCHWA-D	NBB	70-75	6
HCHWA-D	NBB	60-64	7
HCHWA-D	LUMC	55-60	6
HCHWA-D	LUMC	55-60	3
HCHWA-D	LUMC	50-54	6
HCHWA-D	LUMC	50-54	3
HCHWA-D	LUMC	50-54	3
HCHWA-D	LUMC	50-54	19
HCHWA-D	LUMC	45-50	11

NDC non-demented control, HCHWA-D hereditary cerebral hemorrhage with amyloidosis-Dutch type, NBB Netherlands Brain Bank, LUMC Leiden University Medical Center, n.a. not available.
[a] Post-mortem delay (in hours)

Figure 1: Patient material overview. Controls without stroke were age-matched [mean age ±standard deviation (SD) HCHWA-D: 55.8 ±7.1; NDC: 58.6 ±8.4]; both gender were included in the two groups (%M, %F; HCHWA-D: 78, 22; NDC: 56, 44); and *post mortem* delay (PMD; in hours) were not significantly different (HCHWA-D: 7.1 ±5.2; NDC: 7.2 ±2.2). Frontal and occipital human *post mortem* brain tissue was obtained from the Netherlands Brain Bank (NBB) and from our hospital (LUMC).

When we analyzed DEG independently of cases, we found 380 genes differently expressed in frontal *vs*. occipital cortex, but in the HCHWA-D cortex we did not find evidence that the two brain regions were differently affected. Therefore, in all further analyses, frontal and cortical samples in each group were pooled (18 versus 18 samples). HCHWA-D whole dataset and subset of significantly altered genes (FDR < 0.05) with both FC and FDR

are provided in Datasheet 4_Supplementary Material 1. 2048 significant DEG were identified (Figure 3B) including 1201 (7.5%) up-regulated DEG and 847 (5.3%) down-regulated DEG. The top list of DEG, ranked on FC, is given in Figure 4A (down-regulated DEG with both FC and FDR) and Figure 4B (up-regulated DEG with both FC and FDR). Top DEG ranked on significance can be found in Datasheet 3_Supplementary Figure 5.
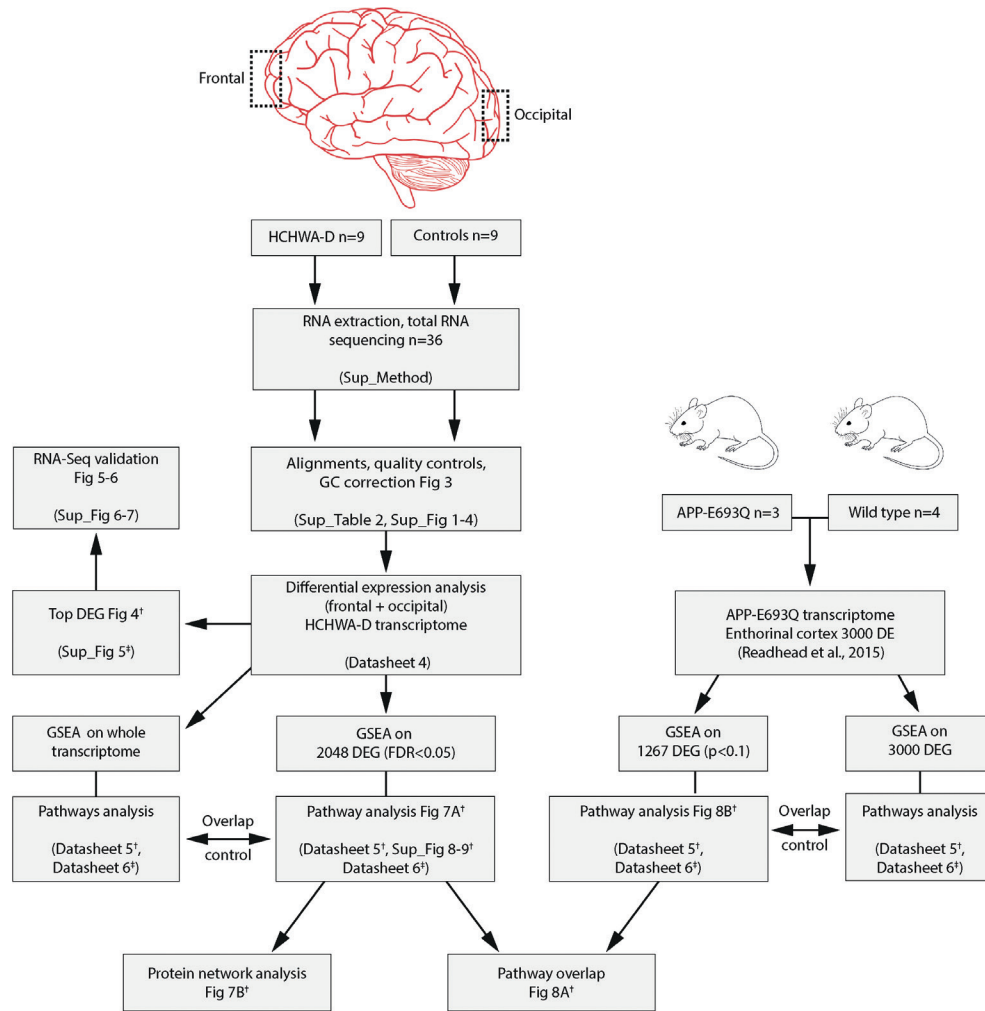
Figure 2: Flow chart of the study and associated files.
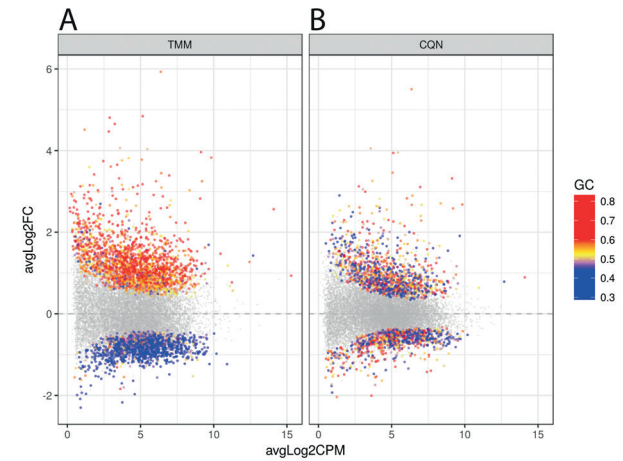
Figure 3: Dot plot of the differentially expressed genes (DEG) in HCHWA-D samples compared to the control samples with log2 fold change (log2FC) versus log2 counts per million (log2CPM) (**A**) without correction and (**B**) with CQN correction for GC bias. Significant DEG in both panels are highlighted by colored dots; colors in legend indicates the GC content.

Validation of top up-regulated and down-regulated genes

Five genes from the top DEG (up- and down-regulated) were selected based on FC, FDR and expression level. Three up-regulated genes (*HSPA1A*, *NPTX2* and *PDYN*) and two down-regulated genes (*GPD1* and *CX3CR1*) were validated by qPCR. The trend for up or down-regulation was confirmed, although statistical significance was not reached for every target principally due to high variability in patient samples (Figure 5). Nevertheless, the correlation of gene expression per patient between the count matrix (log2CPM) and the qPCR was highly significant for *NPTX2*, *PDYN* and *GPD1* and significant for *HSPA1* and *CX3CR1* (Datasheet 3_ Supplementary Figure 6).

For protein level validation of these five genes, western blots were performed on brain homogenates. HSP70 protein was found to be up-regulated in occipital cortex of HCHWA-D, although the difference was not significant (Figure 6; original picture in Datasheet 3_Supplementary Figure 7).

Pathway analysis

We performed GSEA in Gene Trail2 on gene sets sorted by FC. GSEA tests per category whether the genes in the set are randomly distributed or accumulate at the top (''enriched'' pathway) or the bottom (''depleted'' pathway) of the sorted input list (14). Accordingly, ''depleted'' pathways

A

Gene name	Log2FC	adj.P.Val	Full name
DRD5	-2.04	1.058E-05	Dopamine Receptor D5
TMEM125	-2.01	0.008	Transmembrane protein 125
TSPAN8	-1.93	0.012	Tetraspanin 8
LOC101927641	-1.77	0.036	Non coding RNA
CX3CR1	-1.74	0.015	Chemokine (C-X3-C motif) receptor 1
LOC403323	-1.73	0.049	Non coding RNA
DNAH17	-1.64	0.004	Dynein, axonemal, heavy chain 17
SUSD3	-1.56	0.009	Sushi Domain Containing 3
GJB1	-1.56	0.047	Gap junction protein, beta 1, 32kDa
GPIHBP1	-1.54	0.015	Glycosylphosphatidylinositol anchored high density lipoprotein binding protein 1
TYMSOS	-1.54	0.015	TYMS Opposite Strand
NAPSB	-1.52	0.010	Napsin B Aspartic Peptidase, Pseudogene
GPD1	-1.51	0.019	Glycerol-3-phosphate dehydrogenase 1

B

Gene name	Log2FC	adj.P.Val	Full name
HSPA6	5.50	3.19E-06	Heat Shock Protein Family A (Hsp70) Member 6
INHBA	4.05	3.19E-05	Inhibin Beta A Subunit
PDYN	3.95	3.63E-04	Prodynorphin
RRAD	3.94	2.70E-04	Ras-related associated with diabetes
TIMP1	3.46	1.80E-04	TIMP metallopeptidase inhibitor 1
HSPA1A	3.32	1.63E-05	Heat shock 70kDa protein 1A
LINC01164	3.25	1.02E-02	Long Intergenic Non-Protein Coding RNA 1164
SECTM1	3.12	1.22E-02	Secreted And Transmembrane 1
HSPB1	3.11	1.75E-06	Heat shock 27kDa protein 1
CHI3L2	3.05	1.39E-02	Chitinase 3-like 2
SPOCD1	2.99	6.22E-03	SPOC domain containing 1
GNG10	2.90	1.11E-02	Guanine nucleotide binding protein (G protein), gamma 10
BAG3	2.90	8.73E-05	BCL2-associated athanogene 3
SERPINH1	2.83	1.85E-04	Serpin Family H Member 1
SERPINE1	2.82	3.76E-03	Serpin Family E Member 1
DNAJB1	2.71	8.13E-07	DnaJ (Hsp40) homolog
NPTX2	2.68	8.69E-03	Neuronal pentraxin II
LAMB3	2.67	1.14E-02	Laminin, beta 3
VEGFA	2.67	2.54E-07	Vascular endothelial growth factor A
PNOC	2.64	1.48E-02	Prepronociceptin
C7orf61	2.64	1.61E-03	Chromosome 7 open reading frame 61
LOXHD1	2.62	1.48E-03	Lipoxygenase homology domains 1
HSPA7	2.61	2.47E-03	Heat Shock Protein Family A (Hsp70) Member 7
GBP2	2.60	1.46E-04	Guanylate binding protein 2
HSPA1B	2.58	3.53E-04	Heat shock 70kDa protein 1B
ADM	2.56	3.20E-05	Adrenomedullin
C2CD4A	2.54	1.10E-02	C2 Calcium Dependent Domain Containing 4A
ACRC	2.51	1.30E-05	Acidic repeat containing

Figure 4: (**A**) Top list of down-regulated genes based on fold change (ranking based on log2FC<-1.5; 13 out of 847 genes shown); (**B**) Top list of up-regulated genes (ranking based on log2FC>2.5; 28 out of 1201 genes shown).
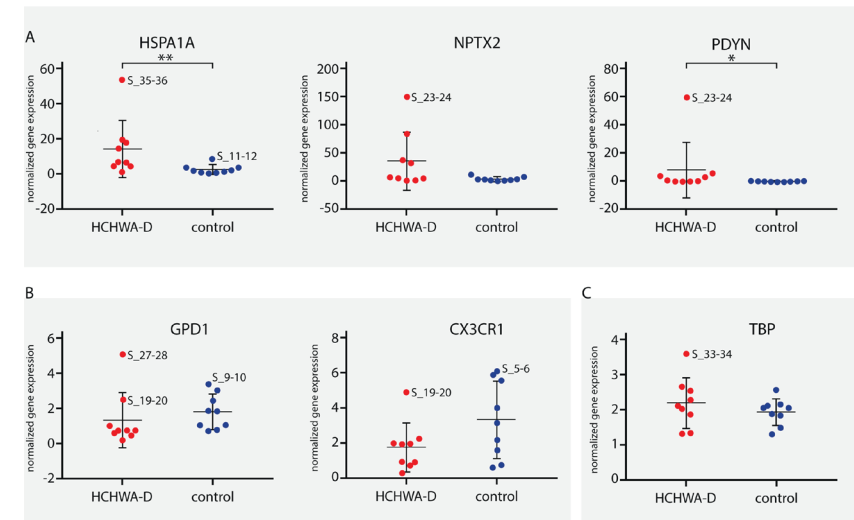
Figure 5: qPCR analysis of (**A**) 3 up-regulated genes (*HSPA1A*; *NPTX2* and *PDYN*) and (**B**) 2 down-regulated genes (*GPD1* and *CX3CR1*). Transcript levels of HCHWA-D samples were not following a normal distribution (data not shown). *HSPA1A* and *PDYN* were found significantly up-regulated (MW test, *p*=0.006** and *p*=0.040* respectively). For *GPD1* significance was reached upon removal of the greatest outlier (*t*-test, *p*=0.030*; H3 outlier identified with ROUT method, Q=1%; data not shown), no significant outliers found for *CX3CR1*. Transcript expression levels were normalized with two reference gene, n=9. (**C**) *TBP* normalization control was not significantly different (*t*-test, *p*=0.32). **p*<0.05, ***p*<0.01.

are overrepresented by down-regulated genes and ''enriched'' pathways are overrepresented by up-regulated genes. Although GSEA doesn't require pre-processed expression data, it has been used on thresholded data, for example to identify robust molecular signatures in tumor diagnosis (15). Similarly, in order to identify predominant pathways and genes dysregulation caused by the APP p.Glu693Gln substitution in HCHWA-D we conducted analysis both on the whole dataset and on the subset of significantly altered genes. Top annotated GO processes were similar in both analyses for the HCHWA-D dataset and for the murine dataset (exhaustive analysis in Datasheet 5_Supplementary Material 2 for genes ranked on FC). The GSEA on genes ranked on FDR is provided in Datasheet 6_Supplementary Material 3. The analysis of the subset of significantly altered genes (ranked on FC) is detailed below.

GSEA on HCHWA-D dataset

GSEA on the significant DEG subset (FDR <0.05; 2036 out of 2048 genes were recognized) was performed. Top annotated GO processes were associated to ''depleted'' mitochondria-related categories and ''enriched'' ECM-related categories. Major dysregulated pathways and identified genes in the DEG subset from Reactome and KEGG are summarized in Figure 7A.

Cellular respiration pathways (oxidative phosphorylation and respiratory electron transport) were ''depleted'' as well as the neurodegenerative diseases pathway in KEGG [Alzheimer disease (AD), Parkinson disease (PD) and Huntington disease (HD)]. As most genes from these pathways overlapped with the cellular respiration (Datasheet 3_Supplementary Figure 8), only the genes non-related to cellular respiration were included in the PD, AD and HD-categories on Figure 7A. Oxidative phosphorylation genes specific for HCHWA-D, i.e. not represented in the other neurodegenerative disease categories were the ATP6V subunits, *COX14*, *COX15*, *LRPPRC* and *TCIRG1*.

ECM-related pathways (ECM-receptor interaction and ECM proteoglycans) were significantly ''enriched'' pathways. Expression values boxplots (Datasheet 3_Supplementary Figure 9) show that many of these genes follow highly concordant trends. Four clusters were defined based on known interactions between genes in this group (Figure 7B). The main cluster (red) includes *CD44* and multiple collagen genes. *CD44* has direct interactions with the three other clusters: integrins and fibronectin (*FN1*) cluster (yellow), lamininB and SV2 cluster (green), and Transforming Growth Factor Beta (*TGFB1* and *TGFB2*), Serpin Family E Member 1 (*SERPINE1*
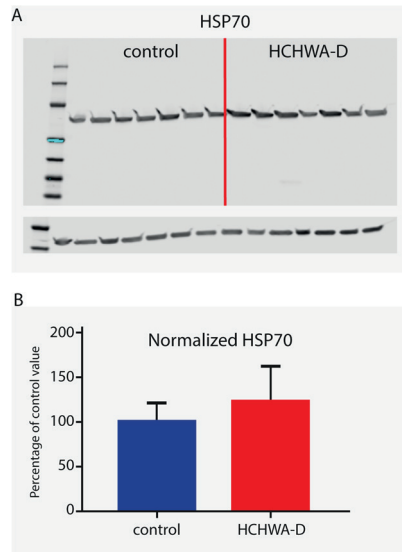

Figure 6: Western blot analysis of HSP70 (antibody detecting *HSPA1A* and *HSPA1B*). (**A**) Signals given by HSP70 antibody showing a band at 70kDa (with β-actin loading control under main blot). (**B**) Signal intensity quantification (n=7, β-actin normalized). Higher protein level in HCHWA-D occipital cortex compared to control occipital cortex was measured although not significantly different; *p*=0.1888 with a two-tailed unpaired Student's *t*-test.
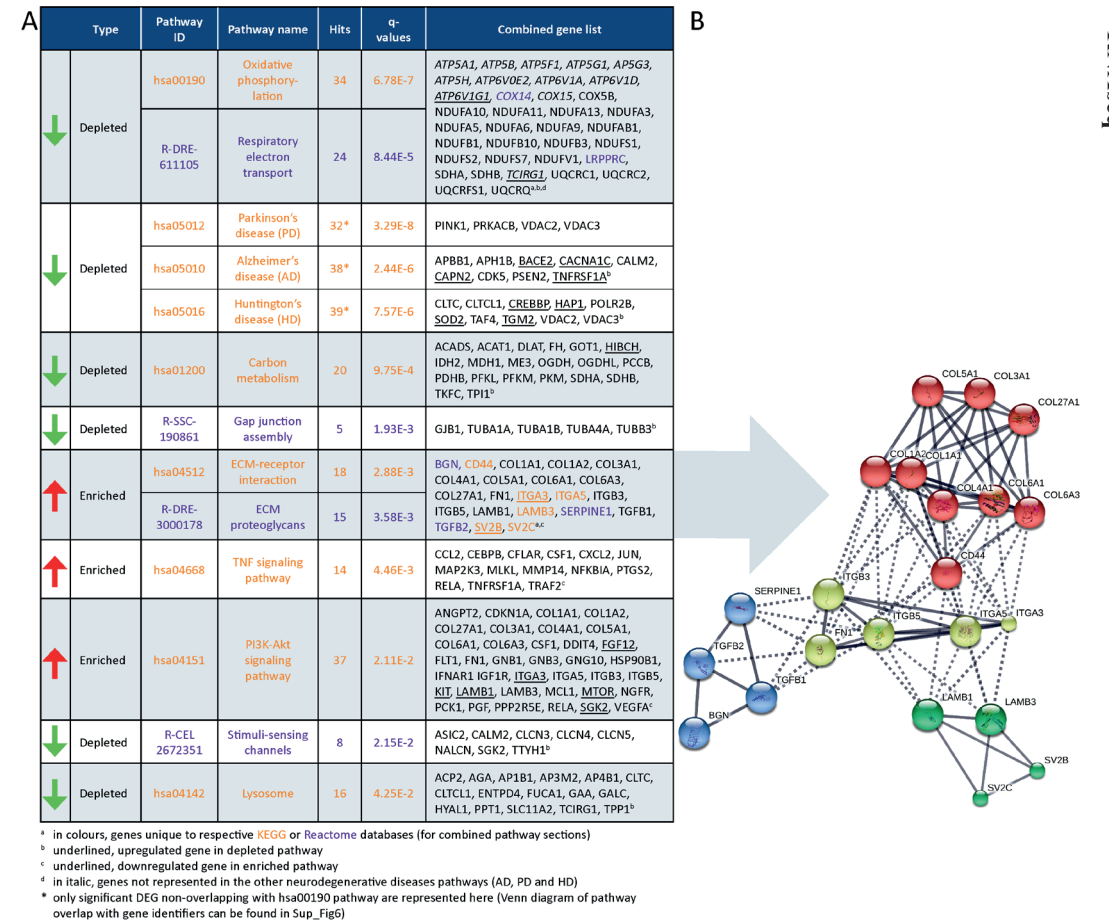

Type	Pathway ID	Pathway name	Hits	q-values	Combined gene list
Depleted ↓	hsa00190	Oxidative phosphorylation	34	6.78E-7	*ATP5A1, ATP5B, ATP5F1, ATP5G1, AP5G3, ATP5H, ATP5V0E2, ATP6V1A, ATP6V1D, ATP6V1G1*, *COX14*, COX15, COX5B, NDUFA10, NDUFA11, NDUFA13, NDUFA3, NDUFA5, NDUFA6, NDUFA9, NDUFAB1, NDUFB1, NDUFB10, NDUFB3, NDUFS1, NDUFS2, NDUFS7, NDUFV1, LRPPRC, SDHA, SDHB, *TCIRG1*, UQCRC1, UQCRC2, UQCRFS1, UQCRQ[a,d]
	R-DRE-611105	Respiratory electron transport	24	8.44E-5	
Depleted ↓	hsa05012	Parkinson's disease (PD)	32*	3.29E-8	PINK1, PRKACB, VDAC2, VDAC3
Depleted ↓	hsa05010	Alzheimer's disease (AD)	38*	2.44E-6	APBB1, APH1B, BACE2, CACNA1C, CALM2, CAPN2, CDK5, PSEN2, TNFRSF1A[b]
	hsa05016	Huntington's disease (HD)	39*	7.57E-6	CLTC, CLTCL1, CREBBP, HAP1, POLR2B, SOD2, TAF4, TGM2, VDAC2, VDAC3[b]
Depleted ↓	hsa01200	Carbon metabolism	20	9.75E-4	ACADS, ACAT1, DLAT, FH, GOT1, HIBCH, IDH2, MDH1, ME3, OGDH, OGDHL, PCCB, PDHB, PFKL, PFKM, PKM, SDHA, SDHB, TKFC, TPI1[b]
Depleted ↓	R-SSC-190861	Gap junction assembly	5	1.93E-3	GJB1, TUBA1A, TUBA1B, TUBA4A, TUBB3[b]
Enriched ↑	hsa04512	ECM-receptor interaction	18	2.88E-3	BGN, CD44, COL1A1, COL1A2, COL3A1, COL4A1, COL5A1, COL6A1, COL6A3, COL27A1, FN1, ITGA3, ITGA5, ITGB3, ITGB5, LAMB1, LAMB3, SERPINE1, TGFB1, TGFB2, SV2B, SV2C[a,c]
	R-DRE-3000178	ECM proteoglycans	15	3.58E-3	
Enriched ↑	hsa04668	TNF signaling pathway	14	4.46E-3	CCL2, CEBPB, CFLAR, CSF1, CXCL2, JUN, MAP2K3, MLKL, MMP14, NFKBIA, PTGS2, RELA, TNFRSF1A, TRAF2[c]
Enriched ↑	hsa04151	PI3K-Akt signaling pathway	37	2.11E-2	ANGPT2, CDKN1A, COL1A1, COL1A2, COL27A1, COL3A1, COL4A1, COL5A1, COL6A1, COL6A3, CSF1, DDIT4, FGF12, FLT1, FN1, GNB1, GNB3, GNG10, HSP90B1, IFNAR1 IGF1R, ITGA3, ITGA5, ITGB3, ITGB5, KIT, LAMB1, LAMB3, MCL1, MTOR, NGFR, PCK1, PGF, PPP2R5E, RELA, SGK2, VEGFA[c]
Depleted ↓	R-CEL 2672351	Stimuli-sensing channels	8	2.15E-2	ASIC2, CALM2, CLCN3, CLCN4, CLCN5, NALCN, SGK2, TTYH1[b]
Depleted ↓	hsa04142	Lysosome	16	4.25E-2	ACP2, AGA, AP1B1, AP3M2, AP4B1, CLTC, CLTCL1, ENTPD4, FUCA1, GAA, GALC, HYAL1, PPT1, SLC11A2, TCIRG1, TPP1[b]

[a] in colours, genes unique to respective KEGG or Reactome databases (for combined pathway sections)
[b] underlined, upregulated gene in depleted pathway
[c] underlined, downregulated gene in enriched pathway
[d] in italic, genes not represented in the other neurodegenerative diseases pathways (AD, PD and HD)
* only significant DEG non-overlapping with hsa00190 pathway are represented here (Venn diagram of pathway overlap with gene identifiers can be found in Sup_Fig6)

Figure 7: Dysregulated pathways in HCHWA-D. (**A**) Significantly dysregulated pathways in HCHWA-D DEG subset and associated genes identifiers in GeneTrail2 v1.5 (with fold change input; ranked on statistical significance except for the combined HD, AD and PD categories). Depleted pathways (green arrow) are overrepresented by downregulated genes and enriched pathways (red arrow) are overrepresented by upregulated genes. Text color code distinguish pathways originating from KEGG (orange) or Reactome (purple) databases. (**B**) Schematic representation of known interactions between genes of the ECM-related pathways. Representation of high confidence interactions with MCL clustering in String interaction database (line thickness indicates the strength of data support).
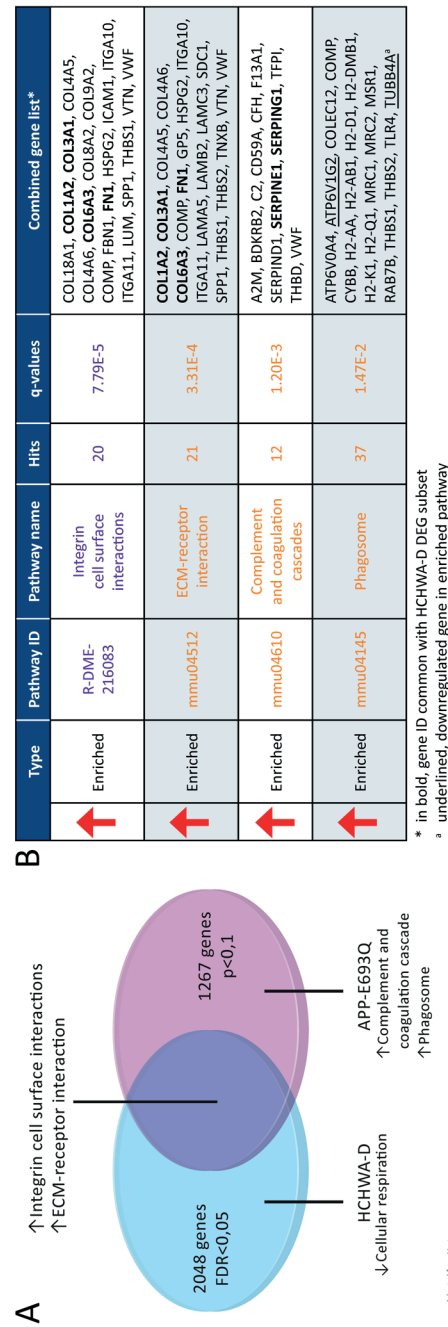
Figure 8: Transcriptome comparison of HCHWA-D and APP-E963Q mouse model (entorhinal cortex; APP-E693Q *vs.* WT; Readhead et al., 2015). (**A**) Venn diagram depicting major dysregulations and overlap in enriched pathway. (**B**) Significantly dysregulated pathways in APP-E693Q DEG subset and associated genes identifiers in GeneTrail2 v1.5 (with fold change input; ranked on statistical significance). Text color code distinguish pathways originating from KEGG (orange) or Reactome (purple) databases.

also known as Plasminogen activator inhibitor-1 or PAI-1) and biglycan (*BGN*) cluster (blue). The integrin and fibronectin group have a central role with interactions with the three other clusters. Additional correlation plots of the gene expression levels per patient between RNA-Seq and qPCR data are given for *TGFB1*, *TGFBR2*, *FN1*, *SERPINE1*, *TIMP-1* and *Col1A1* profibrotic genes (Datasheet 3_Supplementary Figure 10).

GSEA on APP-E693Q dataset and pathways comparison
Murine DEG (entorhinal cortex; APP-E693Q *vs.* WT (10)) were extracted and GSEA on a DEG subset ($p < 0.1$; 1088 out of 1267 genes were recognized) was performed. A transcriptomic comparison between HCHWA-D patients and APP-E693Q mice at the pathway level is schematically shown in Figure 8A. Major dysregulated pathways and identified genes in the APP-E693Q subset are summarized in Figure 8B.

Discussion

Our study provides a comprehensive transcriptome analysis of human HCHWA-D brain cortex. Using RNA-Seq, we identified oxidative phosphorylation dysfunction and enrichment in ECM-related pathways as major transcriptomic changes in HCHWA-D and we revealed an overlap in affected pathways with the APP-E693Q mouse model.

Homogeneity in gene expression between cortical regions
In the current report, we did not find evidence that frontal and occipital cortex of HCHWA-D were differently affected. This finding is in agreement with our previous study where we found no difference in CAA severity between occipital and frontal lobes, both area being similarly severely affected at pathological examination (5).

RNA quality in HCHWA-D group and corrections
Although the RIN values on average were lower in the HCHWA-D group, we did not observe an effect of the RIN value on the number of reads, and therefore no correction for RIN value was applied. This is supported by a study of human *post mortem* brain *ex vivo* degradation (mimicking PMD) where correcting for RIN value in data analysis did not remove the induced degradation bias (16). Nevertheless, a GC bias positively correlated with the median 5'-3' bias, and affecting the FC after normalization, was corrected. Additionally, GC-content of genes might be associated with RNA decay and genes playing regulatory function were found more unstable (16–18). Alternatively, lower RNA quality was recently associated with dementia

diagnostic (19), also occurring in HCHWA-D (20) (unknown dementia status here). Accordingly, an accelerated RNA degradation in HCHWA-D samples could reflect underlying pathogenesis and brain damage. We are confident that the GC correction rectified possible RNA integrity differences as we found a very high correlation with qPCR on random-primed cDNA, a technique that suffers much less from RNA degradation and GC bias.

Oxidative phosphorylation dysfunction in HCHWA-D

*C*ellular aerobic respiration*,* including ATP synthesis and carbon metabolism (TCA cycle, glycolysis/gluconeogenesis) were most significantly altered pathways, dominated by down-regulated genes, which indicates a mitochondrial dysfunction in HCHWA-D.

The mitochondrial respiratory chain complex I (NADH-Ubiquinone) and the ATP synthase complex V (F-type ATPase) were altered pathways in HCHWA-D like in other neurodegenerative diseases (AD, PD and HD). This cellular energy dysfunction is a common denominator in neurodegenerative diseases (21,22). Mostly studied in AD, in the context of neuronal mitochondrial dysfunction (23), oxidative injury has also been studied in endothelial and perivascular cells (24). In particular, *in vitro* studies have shown that especially the Dutch-Aβ-peptide (AβE22Q), via accelerated generation of toxic oligomeric species, could induce mitochondrial alteration of smooth muscle and endothelial cells, leading to induction of apoptosis (25). Although confirmed in the APP-E693Q mice, the presence of oligomeric Aβ species in HCHWA-D has not been demonstrated yet.

The vacuolar proton pumps (V-type ATPase), responsible for acidifying the vacuolar system and in particular in the phagosomal/lysosomal vesicles, was also part of the oxidative phosphorylation pathway affected in HCHWA-D (ATP6V subunits and *TCIRG1*). Since vacuolar proton pumps have a high ATP-demand, mitochondrial dysfunction thus could also impacts autophagocytosis and protein turnover, thereby contributing to pathogenic protein accumulation (26).

Enrichment in ECM-related pathways in HCHWA-D

In contrast, genes in the ECM-related pathways were mostly up-regulated in HCHWA-D brain samples. We showed differential expression of *CD44*, a cell-surface glycoprotein that acts as a principal receptor for ECM proteins such as hyaluronic acid, osteopontin, collagens and mediates the cell surface activation of matrix metalloproteinase. *CD44* has been implicated in several inflammatory diseases (27), as well as in AD pathology where an increase in reactive CD44-positive astrocytes was demonstrated (28). In addition, CD44v6 and CD44v10 splice variants were significantly higher in AD hippocampal neurons (29), and in lung fibrosis, CD44v6 is mediating

the induction of *COL1* pro-fibrotic action of TGFβ1 (30). In HCHWA-D, upregulation of *FN1*, *SERPINE1* and collagen genes also suggests TGFβ1-mediated fibrosis, as proposed in our recent study (5). The presence of *TIMP-1* among the top 5 up-regulated genes strengthens this hypothesis. *TIMP-1* is a strong inhibitor of matrix metalloproteinase thereby reducing ECM turnover and exacerbating the fibrosis. Of interest, *FN1*, which was commonly up-regulated in HCHWA-D and APP-E693Q mice, was also found up-regulated in plasma of both asymptomatic HCHWA-D and *PSEN1* mutation carriers (31) and was recently proposed as a blood-based biomarker for AD (32). Additionally, basement membrane thickening of the vessel wall by *FN1* was shown in early stages of AD before Aβ deposition (33). The use of ECM component detection in plasma, like *FN1*, as biomarkers to monitor early CAA-associated changes in the cerebrovasculature requires further investigation. Of therapeutic relevance, *PSTG2* (COX-2) inhibitor celecoxib was found to lower *FN1* and *COL1* expression and attenuate the vessel wall thickness in stroke-prone spontaneously hypertensive rats (34).

Other identified pathways in HCHWA-D

APP expression was not up-regulated, but some specific APP cleavage enzyme genes were altered, indicating disturbances in APP processing. *APHB1* and *PSEN2*, both functional component of the gamma-secretase complex (cleavage at Aβ C-terminal side) were down-regulated, possibly also affecting the Notch-signaling. *BACE2* (cleavage at β-site and within Aβ region), on the other hand was up-regulated. Aβ production by *BACE2* was increased by the Flemish mutation (p.AlaA692Gly) and *BACE2* is highly expressed in the vasculature (35). These findings are in accordance with earlier studies suggesting that APP-Dutch mutation pathogenic effect was due to an altered APP processing more than a global increase in Aβ production (36–39).

The inflammation component although not dominating, was represented in the TNF-signaling pathway, TNF being a potent pro-inflammatory cytokine. In particular the leukocytes activation and recruitment genes (*CCL2*, *CXCL2*, *CSF1*) are present and could indicate a vascular inflammation and endothelial cell activation, similarly to AD dysregulation (40). PI3k/Akt signaling, also part of the TNF super-family, is involved in AD affecting endothelial cell viability and angiogenesis (41).

Lastly, the Heat Shock Proteins (HSPs) family was highly represented in the top up-regulated genes, in particular HSP70 (HSPA family). HSP are commonly up-regulated in AD (42) but also in PD, PSP and FTLD-Tau (43). They are central to many mechanisms and likely involved in response to ER stress and protein misfolding but can also have an anti-apoptotic role (via JNK, MAPK, ERK and PARP-1 signaling) and modulate ECM (via focal

adhesion and Akt signaling). Small HSPs (sHSP, HSPB family) contribute to neuropathology by actively triggering inflammatory reactions in AD and in HCHWA-D (44,45). Noticeably the top up-regulated sHSPs in our study HSP27 (*HSPB1*) and αB-crystallin (*CRYAB*), were not associated with CAA in HCHWA-D but were already suggested to be efficient chaperone maintaining the peptide in an oligomeric state not trapped into aggregates (45,46).

Overlap with APP-E693Q mouse model pathways

We compared transcriptome changes in HCHWA-D human brain tissue and the APP-E693Q mouse model in order to isolate mutation-associated primary dysregulation with the mouse model representing an early stage of the disease before CAA appearance, while the human *post mortem* samples are the end stage of the disease. The identified ECM-related pathway overlap is striking.

Genes involved in cellular aerobic respiration were not affected in the transcriptome of 12-month APPE-693Q mice but phagosomal system genes were predominantly up-regulated, possibly indicating an early compensatory mechanism to remove the excess of toxic proteins. Consistently, an age-dependent lysosomal dysfunction was recently described in the APP-E693Q mice, with a higher lysosomal count in the entorhinal cortex of the 12-months mice and autophagosomal/autolysosomal protein level increases only in 24-month old mice, leading to an inflammatory reaction and neuronal loss (47). In HCHWA-D the lysosomal pathway was significantly down-regulated probably representing an end-stage state of the disease.

Although we did not find a significant overlap in the complement and coagulation cascades pathway, *SERPINE1* and *SERPING1* were commonly up-regulated in HCHWA-D and APP-E693Q indicating an early altered balance between thrombosis and fibrinolysis. *SERPING1* (C1-inhibitor) is controlling complement activation, blood coagulation, fibrinolysis and generation of kinins. C1-inhibitors are neuroprotective after ischemic stroke (48) and traumatic brain injury (49) via important anti-inflammatory and antithrombotic mechanisms. On the other hand, *SERPINE1* plays a major role in pro-thrombotic conditions, regulating tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA) activity. *SERPINE1* gene expression was found up-regulated in AD brain (7) as well and could be a plasmatic biomarker for the early detection and diagnosis of AD (50).

To conclude, an increase in ECM-related pathways was identified in HCHWA-D and could be, based on the APP-E693Q mice, involved in an early dysregulation inducing pro-fibrotic mechanisms. The mitochondrial dysfunction in HCHWA-D might be a consequence of impaired lysosomal/phagosomal function, in link with the proteinopathies. Alternatively, the

formation of toxic oligomeric Aβ species described in the APP-E693Q mice could trigger oxidative stress affecting the mitochondrial compartment. The formation of toxic oligomeric Aβ species in HCHWA-D and their role of oxidative stress on neuronal, vascular and perivascular cell, would require further investigations.

Conflict of interest

The authors declare no conflict of interest.

Author Contributions

LGM,WR, LW, SK, HM, HB and JL designed the experiments. LGM, LVG and EM performed the experiments. HB supervised the sequencing. HM and SK processed, controlled and generated the datasets. LGM interpreted the datasets and wrote the manuscript. WR and LW supervised and co-wrote the manuscript. KH, SD, MB, PH and SM critically revised the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

Supplementary Material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnagi. 2018.00102/full#supplementary-material

Data availability

Sequence data and count table generated during the current study have been deposited at the European genome-phenome Archive (EGA, http://www.ebi.ac.uk/ega/) which is hosted at the European Bioinformatics Institute (EBI) under EGA accession number EGAD00001003806. Data are available upon approval of the Data Access Committees (EGAC00001000771).

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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

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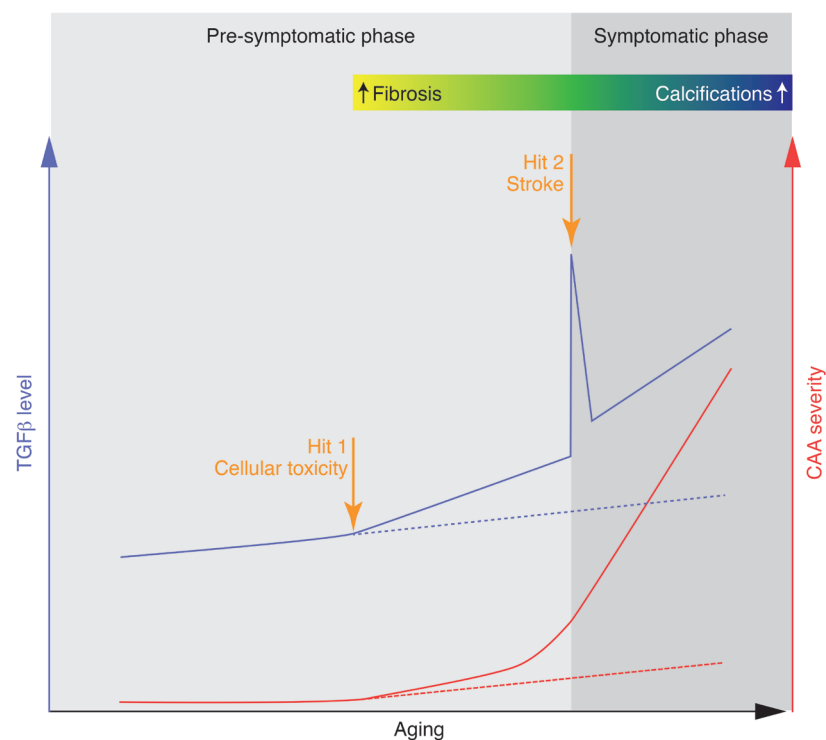
Graphic summary: hypothetical pathomechanisms linking brain TGFβ increase with CAA progression. An early response to cellular toxicity could initiate the TGFβ increase (Hit 1) and trigger the vascular fibrosis in the pre-symptomatic phase. Acute stroke-related TGFβ increase (Hit 2) could further accelerate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

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Summary

Hereditary cerebral hemorrhage with amyloidosis–Dutch type (HCHWA-D) is an early onset hereditary form of Cerebral amyloid angiopathy (CAA) caused by a point mutation of the Amyloid Precursor protein (APP). CAA refers to the accumulation of amyloid β (Aβ) peptide, resulting from APP protein cleavage, in intracerebral vessels. CAA pathology is present in the majority of Alzheimer's disease (AD) brains and is associated with intracerebral hemorrhages in the elderly. The general aim of this thesis is 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.

Chapter 1 is a general introduction of HCHWA-D covering the clinicopathological and neuropathological aspects of the disease as well as the known pathogenesis and the importance of HCHWA-D studies for the CAA field. **Chapter 2** reviews other hereditary APP mutations and describes how the Aβ mutation of HCHWA-D patients modifies Aβ properties regarding aggregation, binding to cerebral vessel wall cells, interplay with extracellular matrix, proteolysis, and clearance, and how these altered characteristics lead to HCHWA-D pathogenesis.

Former research in the CAA field indicates the existence of factors able to influence Aβ accumulation predominantly in vessels rather than in brain parenchyma, such as TGFβ. Identification of aggravating factors of CAA pathology are therapeutic target of interest and therefore we explored in **Chapter 3** the TGFβ pathway deregulation in HCHWA-D. We discovered that in HCHWA-D brain material TGFβ1, its receptors and TGFβ-induced genes are upregulated. Using histology and compared to sporadic CAA, the actual activation of the TGFβ signaling cascade in the hereditary form is confirmed by the accumulation of its activated transcription factor phospho-SMAD2/3 (pSMAD2/3) in some angiopathic vessels which also correlates with the disease severity.

Understanding the underlying pathology of neuroimaging features in patients is essential for a precise diagnostic and follow-up of the disease. Therefore we investigated a recently described MRI neuroimaging feature of advanced disease stage in HCHWA-D. We used an histopathologic correlates in **Chapter 4** to reveal that cerebrovascular iron accumulation and calcification are the cause of the *in vivo* observed MRI abnormalities. We identified calcifications as a novel MRI marker of interest for clinical CAA severity evaluation in hereditary as well as sporadic CAA patients.

Because calcifications are a feature of severe CAA pathology (Chapter 4) and CAA severity is linked with TGFβ deregulation (Chapter 3), we investigated in **Chapter 5** the calcified CAA vessels in HCHWA-D with a focus on TGFβ-related disease progression. Besides pSMAD2/3 immunomarker, we quantified in patients with different severity the presence of osteopontin (OPN) and collagen 1 (col1), potential modulators of vascular calcification responsive to TGFβ. We described an accumulation of OPN and pSMAD2/3 in calcified CAA vessels as well as the association of col1 protein, a fibrotic protein with vascular amyloid load. Chapter 5 tightly link the TGFβ pathway deregulation to CAA progression and calcification in HCHWA-D.

Finally, **Chapter 6** is an transcriptomic study in human *post mortem* brain to identify major deregulated pathways in HCHWA-D and broaden our comprehension of molecular pathogenesis. By comparing the changes that we found in 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 and suggests an early TGFβ1-mediated fibrosis. In addition, we identified a mitochondrial dysfunction, a feature of neurodegenerative diseases not yet described in HCHWA-D.

The discussion in **Chapter 7** summarizes the main finding of this thesis namely that TGFβ deregulation plays a central role in HCHWA-D pathogenesis. Therefore the beneficial and detrimental aspects of TGFβ on the vascular and parenchymal brain components are reviewed and the possible causes of TGFβ activation in HCHWA-D as well as its implication for future studies and therapeutic intervention are discussed.

Nederlandse samenvatting

Hereditary cerebral hemorrhage with amyloidosis (Dutch) (HCHWA-D) is een erfelijke vorm van cerebrale amyloid angiopathie (CAA) die relatief vroeg in het leven ontstaat en veroorzaakt wordt door een puntmutatie in het amyloid precursor eiwit (APP). Doordat APP door enzymen wordt geknipt, wordt het kleinere amyloid β (Aβ) eiwit gevormd. Bij CAA hoopt dit eiwit op in bloedvaten in de hersenen. Naast deze erfelijke vorm is CAA ook aanwezig in de meeste patiënten met de ziekte van Alzheimer. CAA wordt gezien als een belangrijke oorzaak van hersenbloedingen bij ouderen. Het doel van dit proefschrift is om de moleculaire pathogenese van CAA in HCHWA-D patiënten in kaart te brengen. Omdat er nog geen behandeling bestaat om deze ziekte te voorkomen of zelfs te vertragen, is het belangrijk om de onderliggende mechanismen van HCHWA-D te begrijpen. Deze kennis kan helpen om nieuwe therapeutische aangrijpingspunten te vinden en biomarkers te onderzoeken die gebruikt kunnen worden om de werking van mogelijke geneesmiddelen te bekijken.

Hoofdstuk 1 is een algemene introductie over HCHWA-D, waarin de klinische, pathologische en neuropathologische aspecten van de ziekte worden beschreven. Daarnaast is gekeken naar wat bekend is over de pathogenese en naar het belang van HCHWA-D studies voor onderzoek naar CAA in het algemeen.

Hoofdstuk 2 geeft een overzicht van andere erfelijke APP-mutaties en beschrijft hoe de mutatie van HCHWA-D-patiënten de Aβ-eigenschappen verandert met betrekking tot aggregatie, binding aan cerebrale bloedvaten, wisselwerking met extracellulaire matrix, proteolyse en klaring, en hoe deze veranderde kenmerken tot de HCHWA-D pathogenese leiden.

Uit eerder onderzoek naar CAA bleek al dat er factoren zijn die er voor zorgen dat Aβ hoofdzakelijk ophoopt in de bloedvaten en niet in het hersenparenchym. Een voorbeeld hiervan is TGFβ. Zulke factoren zijn interessante therapeutische targets en daarom hebben we in **hoofdstuk 3** de deregulatie van de TGFβ cascade in HCHWA-D onderzocht. In HCHWA-D breinmateriaal hebben we gevonden dat TGFβ1, de receptoren hiervan en TGFβ-geïnduceerde genen zijn verhoogd. De activatie van de TGFβ cascade in erfelijke CAA blijkt ook uit de ophoping van de geactiveerde transcriptiefactor phospho-SMAD2/3 (pSMAD2/3) in sommige angiopathische bloedvaten, wat ook correleert met de ernst van de ziekte.

Het begrijpen van de onderliggende pathologie van kenmerkende verschijnselen op MRI beelden van patiënten zijn essentieel voor een goede diagnostiek en het volgen van de ziekte over de tijd. Daarom hebben we een recent beschreven MRI verschijnsel van een gevorderd stadium van de ziekte in HCHWA-D onderzocht. Hiervoor hebben we in **hoofdstuk 4**

histopathologische correlaties gebruikt om aan te tonen dat cerebrovasculaire ijzerophoping en calcificatie de oorzaak zijn van veranderingen in de MRI-beelden van patiënten. Hierdoor zijn calcificaties een nieuwe interessante MRI-marker voor evaluatie van de ernst van CAA in erfelijke, maar ook in sporadische CAA patiënten.

In **hoofdstuk 5** hebben we de mechanismen onderzocht van de calcificatie van CAA vaten in HCHWA-D. In patiënten met verschillende stadia van CAA hebben we de aanwezigheid van osteopontin (OPN) en collageen 1 (col1) gekwantificeerd. Dit zijn potentiële regelaars van calcificatie in de bloedvaten, bekend van perifere vasculaire calcificatie. De ophoping van OPN en col1 is sterk geassocieerd met pSMAD2/3 in gecalcificeerde CAA vaten, waardoor calcificatie nu gelinkt kan worden aan de eerder gevonden deregulatie van de TGFβ pathway.

Tenslotte hebben we in **hoofdstuk 6** een transcriptoom studie gedaan om belangrijke ontregelingen van moleculaire routes in HCHWA-D te identificeren en de pathogenese beter te begrijpen. Door het menselijk transcriptoom te vergelijken met dat van jonge APP-E693Q muizen op het niveau van ontregelde moleculaire routes, konden we een overlap vinden in de activering van routes die gerelateerd zijn aan de extracellulaire matrix. In muizen zijn deze routes al bij het begin van de CAA pathologie afwijkend, wat suggereert dat TGFβ1-gemedieerde fibrose een vroeg proces is in het verloop van de ziekte. Daarnaast hebben we gezien dat mitochondriale disfunctie een belangrijk ziekteverschijnsel is, wat nog niet eerder beschreven was in HCHWA-D.

De discussie in **hoofdstuk 7** geeft een samenvatting van de belangrijkste bevindingen in dit proefschrift, namelijk dat TGFβ-verstoring een centrale rol speelt in de HCHWA-D pathogenese. Daarom worden de gunstige en nadelige aspecten van TGFβ op de vasculaire en parenchymale hersencomponenten besproken en worden de mogelijke oorzaken van TGFβ-activering in HCHWA-D en de implicatie daarvan voor toekomstige studies en therapeutische interventie besproken.

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List of abbreviations

AD	Alzheimer's disease
ApoE	Apolipoprotein E
APP	Amyloid precursor protein
Aβ	Amyloid beta
ARIA	Amyloid-related imaging abnormalities
BACE	Beta-secretase
BOLD fMRI	Blood-Oxygen-Level Dependent functional MRI
BSA	Bovine serum albumin
CAA	Cerebral amyloid angiopathy
CADASIL	Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy
capCAA	Capillary cerebral amyloid angiopathy
CARASIL	Cerebral autosomal recessive arteriopathy with subcortical infarcts and leukoencephalopathy
CNS	Central nervous system
Col	Collagen
CR1	Complement component receptor 1 gene
CSF	Cerebrospinal fluid
DAB	3,3'-Diaminobenzidine
DEG	differentially expressed gene
E	Acid glutamic
ECM	Extracellular matrix
ECs	endothelial cells
EGCG	Epigallocatechin-gallate
ER	Endoplasmic reticulum
FC	Fold change
FDR	False discovery rate
FN1	Fibronectin
FN1	Fibronectin
GAG	Glycosaminoglycan
GFAP	Glial fibrillary acidic protein
Gln	Glutamine
Glu	Acid glutamic
GSEA	Gene Set Enrichment Analysis
HCHWA-D	Hereditary cerebral hemorrhage with amyloidosis-Dutch type
HD	Parkinson disease

HE	Hematoxylin & eosin		TR	Repetition time
HRP	Horseradish peroxidase		TSA	Tyramide Signal Amplification
HSP	Heat Shock Protein		tTG	Tissue transglutaminase
HSPG	Heparan sulphate proteoglycan		TUDCA	Tauroursodeoxycholic acid
ICH	Intracerebral hemorrhages		uPA	Urokinase-type plasminogen activator
LOX	Lysyl oxidase		VSMCs	Vascular smooth muscle cells
MGE	Multiple Gradient Echo		WMHs	White matter hyperintensities
MMP	Matrix Metalloproteinase		μCT	Micro computed tomography
MRI	Magnetic resonance imaging			
NBB	Netherlands Brain Bank			
NDC	Non-demented control			
NVU	Neurovascular unit			
oAβs	Oligomeric Aβ species			
OPN	Osteopontin			
PAI-1	Plasminogen activator inhibitor-1			
PCA	Principal component analysis			
PD	Parkinson disease			
PiB	Pittsburgh compound B			
PMD	*Post mortem* delay			
PS	Presenilin			
pSMAD2/3	phosphoSMAD2/3			
pTau	Hyperphosphorylated Tau			
Q	Glutamine			
qPCR	Quantitative reverse transcriptase-PCR			
RIN	RNA integrity number			
rRNA	Ribosomal RNA			
RT	Room temperature			
sCAA	Sporadic Cerebral amyloid angiopathy			
SD	Standard deviation			
SMAD	Homolog of Drosophila mothers against decapentaplegic			
SMA	Smooth muscle actin			
SMC	Smooth muscle cells			
SPP1	Secreted phosphoprotein 1			
T	Tesla			
TE	Echo time			
TGFBRs	TGFβ receptors			
TGFβ	Transforming growth factor β			
TOMM40	Translocase of outer mitochondrial membrane 40			

Appendices

Curriculum vitae

Laure Grand Moursel was born on the 18th of March, 1979, in Angouleme, France. She completed her education in France (Master of science degree at Paris 7 University in 2003) and worked five years at Biomethodes (Evry, France), a biotechnology company specialized in the directed evolution of proteins. In 2009 she moved to the Netherlands where she started to work at the Leiden University Medical Center, in the Proteomics group of professor Silvère van der Maarel (Human Genetics department) where she specialized in the development of Heavy Chain antibodies (also known as VHH). In January 2013, she started a PhD in Neurosciences with a focus on Hereditary cerebral hemorrhage with amyloidosis–Dutch type (HCHWA-D) under the combined guidance of associate professor Louise van der Weerd (Radiology department) and associate professor Willeke van Roon-Mom (Human Genetics department) and under the lead of Professor Mark van Buchem, MD (Chair of Radiology department). In November 2018, she started as a senior scientist at Charles River Laboratories in Leiden.

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